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Primary compounds of catalase and peroxidase. By A. S. BRILL and R. J. P. WILLIAMS.

Studies in detoxication. 85. The metabolism of *m*-dinitro[^{14}C]benzene in the rabbit. By D. V. PARKE.

Metabolically inert proteins of the central and peripheral nervous system, muscle and tendon. By A. N. DAVISON.

The location of carbohydrases in the digestive tract of the pig. By A. DAHLQVIST.

Thermodynamic quantities for the dissociation equilibria of biologically important compounds. 8. The first and second acid dissociations of arginine. By S. P. DATTA and A. K. GRZYBOWSKI.

Plant polyphenols. 3. Flavonoids in genotypes of *Primula sinensis*. By J. B. HARBORNE and H. S. A. SHERRATT.

The intracellular distribution of fatty acids in rat liver. The fatty acids of intracellular compartments. By G. S. GETZ and W. BARTLEY.

Determination of inorganic sulphate in studies on the enzymic and non-enzymic hydrolysis of carbohydrate and other sulphate esters. By K. S. DODGSON.

Potassium glucose 6-*O*-sulphate as a substrate for glycosulphatase. By K. S. DODGSON and A. G. LLOYD.

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Studies on pituitary adrenocorticotrophin. 3. Identification of the oxidation–reduction centre. By M. L. DEDMAN, T. H. FARMER and C. J. O. R. MORRIS.

Comparative studies of 'bile salts'. 13. Bile acids of the leopard seal, *Hydrurga leptonyx*, and of two snakes of the genus *Bitis*. By G. A. D. HASLEWOOD.

The breakdown of chlorophyll by chlorophyllase. By M. HOLDEN.

Pyridoxamine phosphate-oxidase and pyridoxal phosphate-phosphatase activities in *Escherichia coli*. By J. M. TURNER and F. C. HAPPOLD.

The sedimentation characteristics of deoxyribonucleic acid from human tissues. By P. A. BIANCHI and K. V. SHOOTER.

Metabolism of polycyclic compounds. 18. The secretion of metabolites of naphthalene, 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene in rat bile. By E. BOYLAND, G. S. RAMSAY and P. SIMS.

Spectrophotometric measurements on ascorbic acid and their use for the estimation of ascorbic acid and dehydroascorbic acid in plant tissues. By E. J. HEWITT and G. J. DICKES.

Studies on the effect of anterior-pituitary growth hormone on oxidative phosphorylation in rat-liver mitochondria. By A. H. MELHUISH and A. L. GREENBAUM.

Some studies on the occurrence of sialic acid in human cartilage. By A. J. ANDERSON.

The preparation and properties of cystine esterase. By K. C. HOOPER.

Studies in the biochemistry of micro-organisms. 108. Metabolites of *Alternaria tenuis* Auct.: the biosynthesis of tenuazonic acid. By C. E. STICKINGS and R. J. TOWNSEND.

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A NEW TEST PREPARATION FOR BIO-ASSAY OF FACTOR I AND GAMMA-AMINOBUTYRIC ACID

BY E. FLOREY

From the Department of Zoology, University of Washington, Seattle 5, Washington, U.S.A.

(*Received* 15 *July* 1960)

During our recent attempts to characterize 'inhibitory' agents (Factor I) in crustacean nerve extracts, we have found that the responses of the isolated hind gut of crayfish can be successfully used for bio-assay of inhibitory actions of nerve extracts, eluates of chromatograms and drugs. Although some pharmacological characteristics of the crayfish intestine have already been described (Florey, 1954*a*, *b*) it appears now worth while to report on the technique of using the isolated intestine preparation for the bio-assay of Factor I activity, and on some newly discovered pharmacological properties. The new method is simpler and more sensitive than that using crayfish stretch receptors.

METHODS

Three species of crayfish have proved equally useful. These were *Procambarus clarkii*, *Orconectes virilis* and *Pacifastacus leniusculus*. The animals should have a minimum body length of 4 in. (10 cm). After the dorsal half shell of the abdomen is removed, the hind gut can be seen as a straight tube which runs along the mid line towards the last abdominal segment. The whole length of the abdominal intestine should be excised. It is advisable gently to empty it of its contents with the aid of a blunt instrument and to flush the lumen with saline medium. The organ should be mounted in a long and narrow muscle chamber. The later performance of the preparation is greatly improved if the gut is filled and slightly distended with saline medium before the second ligature is tied. The proximal end of the gut is then connected to a well balanced light lever so as to give a tenfold magnification of the contractions. The movements of the intestine are recorded on the smoked drum of a slow-moving kymograph. Only glossy kymograph paper should be used, in order to offer minimal frictional resistance to the writing stylus.

A saline medium of the following composition has been found suitable (g): NaCl 12·0, KCl 0·4, $CaCl_2$ (anhydrous) 1·5, $MgCl_2.6H_2O$ 0·5, H_2O to 1000 ml. No buffer is needed, but the muscle bath should be aerated with a constant stream of fine air bubbles to achieve oxygenation and rapid mixing of the bathing media. Organ and solutions are held at room temperature.

Before application of test samples, these have to be freed from all traces of organic solvents and any acid or base must be neutralized. Although it is advisable to make the samples isotonic with the saline medium used, by adding 1/9 volume of 10 times concentrated saline stock solution (K-free where necessary), this is not very important because the samples are

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Before application of test samples, these have to be freed from all traces of organic solvents and any acid or base must be neutralized. Although it is advisable to make the samples isotonic with the saline medium used, by adding 1/9 volume of 10 times concentrated saline stock solution (K-free where necessary), this is not very important because the samples are

usually so greatly diluted in the bath that the change in the osmotic concentration of the bathing medium does not exceed 10%.

Before the addition of samples to the bath the volume of the latter is reduced to 9/10 of the standard level. An amount of the sample is then added to bring the bath up to the original level. In our tests we use a total bath volume of 2 ml., the sample volumes added are therefore 0·2 ml.

Before the test proper one has to find a concentration of acetylcholine which will cause a marked but submaximal increase in 'spontaneous' contractions and a definite rise of the base line. A concentration of acetylcholine at 10^{-7} g/ml. bathing medium was found adequate in most tests.

The test for inhibitory action of samples consists in the application of suitable concentrations of a test sample and subsequent addition of acetylcholine. Reduction of spontaneous contractions and depression of the response to acetylcholine are criteria for inhibitory actions.

RESULTS

The actions of Factor I, amino acids and picrotoxin

Factor I has originally been defined as that agent in nerve extracts which is responsible for inhibition of impulse generation in the slow-adapting neurone of the abdominal stretch receptor organs of crayfish (Florey, 1954*a*). It has later been shown that several substances occurring in nerve tissue can inhibit the stretch receptor neurone. Some of these are known: beta-alanine, guanido-acetic acid, gamma-aminobutyric acid, beta-hydroxy-gamma-aminobutyric acid, gamma-guanidinobutyric acid and gamma-aminobutyro-choline (Bazemore, Elliott & Florey, 1957; McLennan, 1959). Other components of Factor I preparations which have inhibitory activity on the stretch receptors are still unknown. One of them is referred to by McLennan as Fraction A of Factor I. A similar compound has been chromatographically isolated from crustacean nerve tissue by Florey & Chapman (1961) who refer to it as Substance I (Florey, 1960). Fraction A and Substance I seem to be identical.

Extracts of vertebrate and crustacean nerve tissue which have inhibitory actions on crayfish stretch receptors depress the spontaneous activity of the isolated crayfish intestine and block the stimulatory action of acetylcholine on this organ. The sensitivity of the intestine to inhibitory agents is greater than that of the stretch receptors, but it can be shown that the inhibitory substances which are effective on the two organs are the same.

Aqueous extracts of brains of rats, rabbits and cattle were found to depress spontaneous and acetylcholine-induced contractions of the crayfish intestine in concentrations up to five times lower than those which show liminal effectiveness on the stretch-receptor preparation. Aqueous and methanol extracts of lobster (*Homarus americanus*) and crab (*Cancer magister*) nerves were found to be 20–25 times as active if tested on the intestine, compared to their action on stretch receptors. By means of paper chromatography (see Florey & Chapman, 1961) it could be demon-

strated that the compounds which inhibit the stretch receptors are the same as those which inhibit the intestine of the crayfish. This is true for components of extracts from nerve tissue of both crabs and mammals. It is difficult to express the activities of the inhibitory components in terms of absolute concentrations of the compounds, since their nature and amount in nerve tissue has not been established (with the exception of certain amino acids which will be dealt with below). It can, however, be stated that the lowest effective concentrations of extracts of mammalian brain (rat, rabbit, cattle) are 10–25 mg fresh tissue/ml. for stretch receptors and 5–10 mg/ml. for isolated intestines. For extracts of crab nerve extracts (*Cancer magister*) the concentrations are 10–25 and 0·5–1 mg/ml. respectively. Details of the experiments involving paper chromatography will be published later.

Gamma-aminobutyric acid was found to inhibit spontaneous and acetylcholine-induced contractions of the crayfish intestine in liminal concentrations of 2×10^{-6} g/ml. Inhibitory actions are produced by a number of other compounds of similar chemical nature. These substances and their liminal concentrations (g/ml.) are: guanido-acetic acid 10^{-6}; glutamic acid, 5×10^{-5}; monosodium glutamate, 5×10^{-5}; beta-hydroxy-gamma-aminobutyric acid, 2×10^{-5}; gamma-aminobutyrylcholine, 5×10^{-5}; beta-alanine, 10^{-4}; aspartic acid, 2×10^{-5}; taurine 10^{-4}.

The following compounds were found to have no detectable action in concentrations as high as 10^{-4} g/ml.; gamma-guanidinobutyric acid; succinylcholine chloride; L-alanine; pyroglutamic acid; acetylglutamic acid; DL-methylglutamic acid; L-histidine; acetylhistidine; DL-phenylalanine; DL-alpha-phenyl-alpha-alanine; L-leucine; DL-acetylleucine; DL-tyrosine; L-cysteine; S-carbamyl-1-cystein; DL-proline; DL-acetylproline; creatine; creatinine; L-tryptophan; cadavarine HCl; canavanine sulphate; sarcosine; carnitine HCl and carnosine.

The following compounds were found to have a stimulatory action, causing an increase in strength and frequency of the contractions at the following liminal concentrations (g/ml.): acetylcholine, 10^{-8}; acetyl-beta-methylcholine, 2×10^{-6}; butyrylcholine, 2×10^{-6}; adrenaline, 10^{-6}; noradrenaline, 10^{-6}; 5-hydroxytryptamine, 2×10^{-6}; picrotoxin, 5×10^{-5}. Atropin in a concentration of 5×10^{-6}–5×10^{-7} g/ml. is an effective blocking agent for acetylcholine, acetyl-beta-methylcholine and butyrylcholine. It does not interfere with the action of the other excitatory compounds. Picrotoxin in concentrations from 10^{-5} to 10^{-6} g/ml. effectively reduces or blocks the action of all inhibitory compounds mentioned, including the chromatographically isolated inhibitory components of nerve extracts. It does not interfere with the action of the excitatory compounds mentioned. In somewhat higher concentrations, such as 5×10^{-5} g/ml.,

picrotoxin has a slight stimulatory action. Atropin if applied in higher concentrations, such as 10^{-5} g/ml., depresses the spontaneous activity of the intestine. Representative responses of the isolated crayfish intestine to nerve extracts and drugs are shown in Fig. 1.

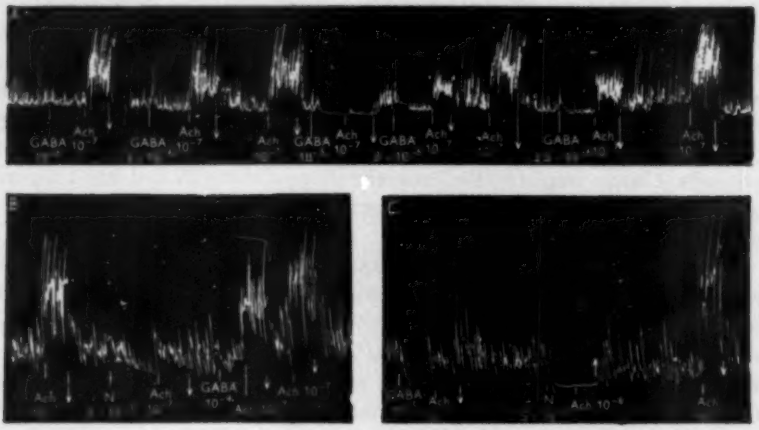

Fig. 1. Responses of an isolated intestine of *Pacifastacus leniusculus* to applied drugs and nerve extract (N). *A*: interaction of gamma-aminobutyric acid (GABA) and acetylcholine (ACh); note the graded response. *B*: onset of the action of picrotoxin which is maintained at a concentration of 10^{-5}g/ml; note that GABA even in high concentration is unable to prevent the action of ACh; the inhibitory action of a nerve extract (N) is partially blocked. *C*: after 15 min washing the inhibitory actions of GABA and N are restored. The record *A* was obtained from a different organ from those of *B* and *C*. The arrows indicate washing. In *B* the washing solution contained picrotoxin.

The method of bio-assay

The isolated crayfish intestine can be used for bio-assay of Factor I activity in the following manner: after a concentration of acetylcholine has been found which causes marked but submaximal contractions, 1/10 volume of the unknown sample is added to 9/10 volume of the bath. Thirty seconds later 1/10 volume of fluid is removed from the bath and 1/10 volume of the appropriate acetylcholine solution is added. One minute later the bath is emptied and the preparation is washed twice with pure saline medium. After an interval of 2 min the volume of the bath is reduced to the 9/10 level and 1/10 volume of the acetylcholine solution, as used before, is added. After 1 min this is followed by a double washing. Two minutes later another dilution of the same unknown can be applied, to be followed 30 sec later by addition of acetylcholine as described above.

Inhibitory action is characterized by the depression of spontaneous

contractions and depression of the acetylcholine action. The inhibitory activity of an unknown sample can be standardized against known concentrations of gamma-aminobutyric acid. For this purpose it is necessary to find a concentration of gamma-aminobutyric acid which will reduce the action of acetylcholine by an amount equal to that achieved with a certain concentration of the unknown. The most economic way to do this is first to find the lowest concentration of the unknown which will still definitely depress the acetylcholine action and then to find a dilution of gamma-aminobutyric acid which will match the effect of the unknown. Once such a concentration of gamma-aminobutyric acid is found, the unknown should be applied once more to make certain that the level of sensitivity of the test organ has remained the same.

Differences in inhibitory activity of plus or minus 20% are readily detectable with this test. The inhibitory activity can be expressed in terms of μg gamma-aminobutyric acid/g fresh nerve tissue. For instance, if an extract diluted to 1:1000 (original nerve tissue 1 mg/1 ml. bath) causes an inhibition comparable to that produced by the application of 10 μg of gamma-aminobutyric acid/ml., 1 g of the original nerve tissue from which the extract was prepared must have contained (at least) as much inhibitory activity as 1000×10 μg of gamma-aminobutyric acid.

At the end of the assay procedure picrotoxin should be added to the saline medium in the bath, to give a final concentration of 2×10^{-5} g/ml. After the picrotoxin solution has been in contact with the preparation for 10 min, gamma-aminobutyric acid and unknowns are applied once more to ascertain the Factor I nature of the unknown. Gamma-aminobutyric acid serves as a control. Both inhibitory actions should be prevented or greatly reduced by the presence of picrotoxin in the bath. The effects of acetylcholine-blocking agents such as atropine are not changed by picrotoxin.

DISCUSSION

Although the bio-assay for Factor I activity by means of the crayfish stretch receptor preparation, as described by Elliott & Florey (1957) and Florey & Elliott (1961), is more precise, the new bio-assay method has the advantage of greater sensitivity and simplicity. Not only is the dissection the easiest possible, but the equipment needed for the tests is more readily available and the assay can be carried out by persons who are not familiar with electronic apparatus.

The physiology of the test organ is, unfortunately, little understood. The intestine is innervated by two large lateral nerve strands, which originate in the sixth abdominal ganglion. The nerves usually remain attached to the intestine when this is removed from the animal. The responses of the intestine are, however, not altered if they are dissected

off. Alexandrowicz (1909) has shown that there are numerous nerve cells within the wall of the intestine. We know that stimulation of the lateral nerves induces a contraction and peristaltic wave, but it is uncertain whether this action is a direct one on the muscle or whether it is mediated by the nerve cells. It is likely that the action of acetylcholine is through stimulation of the nervous elements of the intestine, for the following reasons: The musculature of the intestine is of the striated type and none of the muscles of the crayfish responds to acetylcholine. Crustacean nerve cells are stimulated by acetylcholine and 5-hydroxytryptamine (Wiersma, Furshpan & Florey, 1953; Florey, 1954*c*). Adrenaline and noradrenaline stimulate the intestine. Atropine blocks the action of acetylcholine but not that of adrenaline or noradrenaline. Factor I and gamma-aminobutyric acid, on the other hand, block the actions both of acetylcholine and of adrenaline and noradrenaline. This means that during atropine action the intestinal musculature is still excitable (by adrenergic agents), while during Factor I action it is not. One could thus conclude that acetylcholine activates non-cholinergic nerve cells which are responsible for the 'spontaneous' activity of the intestinal musculature, and that Factor I as well as gamma-aminobutyric acid acts at the neuromuscular junctions and—quite likely—on the nerve cells as well. Since Factor I and gamma-aminobutyric acid prevent the excitatory action of adrenaline and noradrenaline, it is very unlikely that their mechanism of action consists in (competitive) inhibition of cholinoceptive receptor molecules.

The relative insensitivity of the intestine towards common amino acids which tend to obscure bio-assays using the crayfish stretch receptor preparation is of great advantage to those interested in the chemical isolation and identification of 'inhibitory' substances. The extraordinary inhibitory action of crustacean nerve extracts, as described in the preceding section, points once more to the fact that gamma-aminobutyric acid is not likely to be the transmitter substance (see Florey, 1960; Florey & Biederman, 1960; Florey & Chapman, 1961).

SUMMARY

1. The spontaneous contractions of the isolated hind gut of crayfish are greatly enhanced by acetylcholine and depressed by certain inhibitory substances that can be isolated from mammalian and crustacean nerve extracts.

2. The agents responsible for the inhibitory actions are identical with those which inhibit impulse generation in the crayfish stretch receptor preparation. The crayfish gut can, therefore, be used to detect compounds with Factor I activity. It is very much more sensitive than the stretch receptor.

3. Substances with Factor I activity (in order of decreasing activity) are gamma-aminobutyric acid, guanido-acetic acid, beta-hydroxy-gamma-aminobutyric acid, aspartic acid, glutamic acid, monosodium glutamate, gamma-aminobutyrylcholine, beta-alanine and taurine. All these block spontaneous contractions and the action of acetylcholine. The inhibitory action of these compounds is prevented by picrotoxin.

4. Compounds are listed which are excitatory and others which are without effect on the crayfish gut.

5. Crustacean nerve extracts contain inhibitory material which appears to be very much more inhibitory than gamma-aminobutyric acid.

6. A method of bio-assay is described which permits quantitative evaluation of Factor I activity using the action of known concentrations of gamma-aminobutyric acid as standards.

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TRANSPORT OF GLUCOSE ACROSS BLOOD–AQUEOUS BARRIERS AS AFFECTED BY INSULIN

BY V. E. KINSEY AND D. V. N. REDDY
From the Kresge Eye Institute, Detroit, Michigan, U.S.A.

(*Received* 8 *August* 1960)

Knowledge of the mode of transport of glucose from plasma to intraocular fluids and its possible dependence upon the action of insulin is important both to an understanding of the normal physiology of intraocular fluids and to the pathogenesis of cataract, which is thought to be associated with diabetes. The idea that insulin plays some role in glucose transport across cell membranes stems in part from experiments of Ross (1952), in which he determined the permeability coefficient for glucose transport across the 'blood–aqueous barrier' from its rate of accumulation in the anterior chamber of rabbit eyes. He reported that this coefficient was reduced by about 50% in rabbits in the hyperglycaemic phase of alloxan diabetes, increased approximately fourfold in animals that were in the hypoglycaemic phase of the disease, and increased threefold in animals given exogenous insulin. These results have been interpreted to mean that insulin increases the rate of transfer of glucose and other sugars through the blood–aqueous barrier of the ciliary body (Stadie, 1954).

The rate of accumulation of a substance in the anterior chamber is dependent not only on its concentration in plasma but also on that in the aqueous of the posterior chamber (Kinsey & Palm, 1955). In calculating permeability coefficients, only the concentration of glucose in the plasma was considered by Ross. Since the concentration in the posterior chamber not only differs from that in plasma but varies with time after injection, the meaning of the coefficients of transport for the anterior chamber, as calculated by Ross, is not readily interpretable. More pertinently, however, because of the dual source of entry of all constituents of the anterior chamber it is not possible, from anterior chamber data alone, to determine accurately the effect of any variable on the permeability of either the iris or ciliary processes. Therefore, more specifically, only limited conclusions can be drawn about the effect of insulin on the permeability to glucose of the barrier separating plasma from anterior chamber, and even more limited conclusions can be drawn concerning the effect this hormone might have on the permeability of the ciliary processes, without knowledge of the rate of accumulation of glucose in the posterior chamber.

The development of a method (Kinsey, 1953) for withdrawing aqueous humour from the posterior chamber in sufficient quantities to perform accurate chemical analyses and the availability of data concerning the rate of flow of aqueous now make it possible to determine the proportionate amount of a substance which flows and that which diffuses into the anterior chamber (Kinsey & Palm, 1955). Moreover, the ability to determine the concentration of a substance in the posterior chamber at any time following parenteral administration also permits the direct evaluation of the effect of a variable on transport rates into the posterior chamber and thus the effect, if any, on the permeability of the ciliary processes.

This paper is concerned with the results of an investigation of the rates of penetration of ^{14}C-labelled glucose into both ocular chambers in normal and diabetic rabbits and in rabbits given exogenous insulin. The mechanism by which glucose gains entry to the posterior and anterior chambers of normal rabbits will be discussed in a later paper.

METHODS

Twenty microcuries of D-glucose uniformly labelled with ^{14}C were given to unanaesthetized albino rabbits (1·8–2·2 kg), 30 % of the dose being given intravenously and the remainder intraperitoneally. Blood and aqueous samples were withdrawn by techniques previously described (Kinsey, 1953) and plated on flat copper planchetets to which 25 μl. of 0·1 N-HCl were added to remove any bicarbonate which might have become labelled. Radioactivity was determined with a thin-window flow-gas detector. Appropriate corrections were applied for self-absorption.

Rabbits were made diabetic by giving single intravenous injections of alloxan (200 mg/kg) and separated into two groups. Four rabbits were studied within the first 48 hr after injection, at which time they were in the hypoglycaemic stage of the disease, and fifteen after hyperglycaemia had become established. Some of these rabbits were studied 5 days after the injection, at which time the plasma and in a few instances the aqueous humour were lipaemic. Other animals were given daily injections of 1–2 u. of insulin from the fourth to the eleventh day after injection of alloxan and studied 4–6 weeks later. The blood-sugar levels of all the animals with established diabetes ranged from 300 to 700 mg/100 ml.

In the experiments concerned with the effect of administration of exogenous insulin, each rabbit was given 2 u. of soluble insulin (Iletin, Eli Lilly) intravenously 60 min before administration of ^{14}C-labelled glucose. The insulin reduced the blood-sugar level in these animals to 25–80 mg/100 ml.

To make certain that all the measured radioactivity came from glucose rather than from some metabolic break-down product, samples of aqueous humour and plasma were taken at intervals up to 30 min after injection of the labelled glucose and subjected to paper chromatography. Phenylhydrazine derivatives of glucose were also prepared from plasma, and the specific activity of the glucose determined. Both these procedures showed that almost all the radioactivity came from glucose.

Some experiments were performed under conditions essentially identical with those employed by Ross, using non-labelled glucose and an anaesthetic (pentobarbitone). In these experiments glucose was given intravenously (solution 10 g/100 ml., 3 ml./kg) and intraperitoneally (20 ml./kg of a solution of 5 g/100 ml. for normal and of a solution of

7·5 g/100 ml. for animals which received exogenous insulin). This mode of administration kept the plasma glucose at a reasonably constant level over the period of the experiment. Glucose was estimated with glucose oxidase (Huggett & Nixon, 1957) on barium hydroxide–zinc sulphate filtrates.

The absolute level of radioactivity found in the blood of different rabbits varied. To permit comparison of the results between animals the concentration per unit volume of each of three plasma samples was plotted as a function of time. The level which most nearly represented the average concentration during the whole period of time was then set equal to 100, and all other plasma and aqueous concentrations from the same animal were scaled accordingly as a percentage of this value. These percentages are referred to as 'relative units'.

In experiments in which blood sugar was elevated by injection of non-radioactive glucose, the initial concentration of glucose was determined in plasma and aqueous samples. Because the driving force is the difference in concentration between plasma and aqueous rather than the absolute magnitude of the concentration, the initial concentration in the aqueous humour was then subtracted from the average of that found in the plasma following infusion. The resulting value was used to determine, on a percentage basis, the increment of concentration in the aqueous found after infusion. Thus, for example,

$$\text{the percentage increase in the posterior aqueous} = \frac{C_{ht_1} - C_{ht_0}}{C_{p_{av}} - C_{ht_0}} \times 100,$$

$$\text{and that in the anterior aqueous} = \frac{C_{at_1} - C_{at_0}}{C_{p_{av}} - C_{at_0}} \times 100,$$

where C_{ht_1} = concentration in the posterior chamber in the contralateral eye at the time when the sample was withdrawn;

C_{ht_0} = concentration in posterior chamber at zero time in one eye;

$C_{p_{av}}$ = average plasma concentration following elevation of blood sugar level by parenteral injection;

C_{at_1} = concentration in the anterior chamber in the contralateral eye at the time when the sample was withdrawn; and

C_{at_0} = concentration in anterior chamber at zero time in one eye.

RESULTS

The concentrations of labelled glucose in the plasma and aqueous humour of the posterior and anterior chambers of untreated rabbits at various times after injection are shown in Fig. 1. The lines are visual fits to the data. Each cross and open circle represents concentration in the aqueous humour of one posterior and one anterior chamber, respectively. Each filled circle shows the concentration in the plasma. Analogous results for diabetic animals in the hyperglycaemic phase of the disease and for animals which were given insulin 1 hr before injection of ^{14}C-labelled glucose are shown in Figs. 2 and 3. A comparison of the data in the three figures shows that the rate of accumulation of glucose in the aqueous of the posterior chamber is substantially the same under all conditions.

The rate of accumulation of glucose in the anterior chamber appears to be slightly lower in diabetic rabbits and slightly higher in rabbits given exogenous insulin than in normal animals. However, considering variations

in the rate of accumulation from one animal to another, differences are of doubtful significance, and in no case do they correspond in magnitude to those reported by Ross.

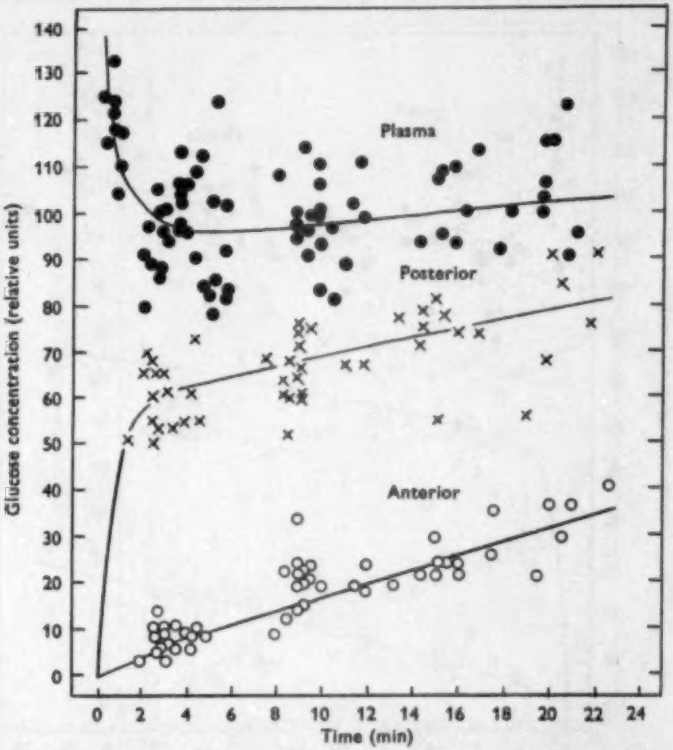

Fig. 1. Relative concentration of ^{14}C-labelled glucose in plasma and aqueous of anterior and posterior chambers in normal rabbits following parenteral administration.

Figure 4 shows the results of observations made on eight eyes of rabbits which were in the hypoglycaemic phase of diabetes. The line through the data representing plasma is drawn as a good visual fit; the lines representing aqueous humour are duplicates of those for untreated rabbits, as shown in Fig. 1. It is evident that the data obtained on aqueous samples taken from animals in the hypoglycaemic phase of diabetes are well represented by curves showing the average analogous data from normal rabbits.

Two sets of experiments were performed to test whether the disparity between the results in the present investigation and those of Ross might

be due to differences in experimental technique employed. First, Ross's procedure was adopted and the rate of accumulation of glucose in both normal animals and those injected with insulin was determined in anaesthetized rabbits with unlabelled sugar. Secondly, the rate of accumulation

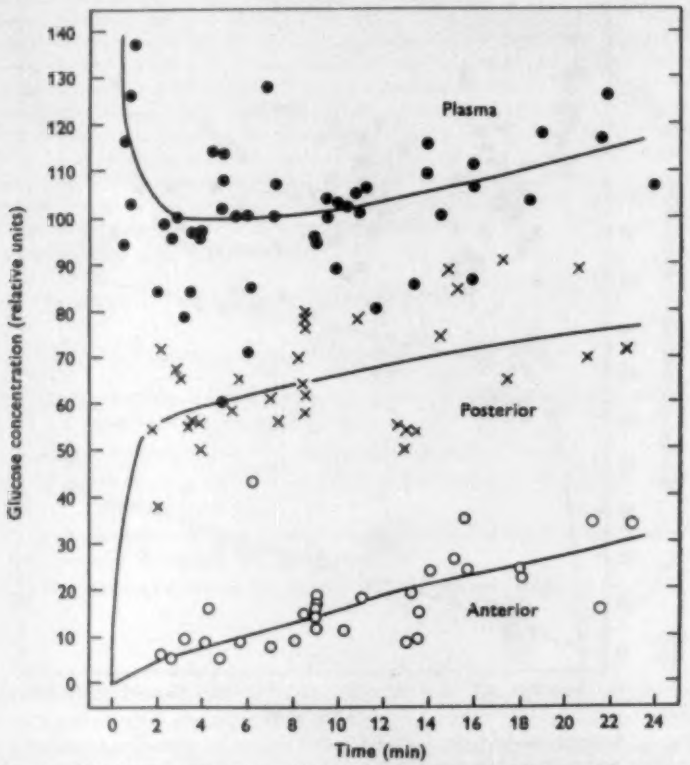

Fig. 2. Relative concentration of ^{14}C-labelled glucose in plasma and aqueous of anterior and posterior chambers in diabetic rabbits following parenteral administration.

of glucose was determined by means of the tracer technique in rabbits in which the blood sugar was first elevated 250–300 mg/100 ml. by parenteral administration of glucose to determine whether the high level of glucose in the blood affected the permeability *per se*. Results obtained for the posterior chamber for both sets of experiments are shown in Fig. 5, and those for the anterior chamber in Fig. 6. Again the lines are taken from Fig. 1.

The results obtained by infusion of unlabelled glucose in normal and

insulin-treated animals, as well as in those with the tracer technique in rabbits whose blood sugar was elevated before giving ^{14}C-labelled glucose, are all essentially the same as those obtained when the tracer technique was employed to study normal animals.

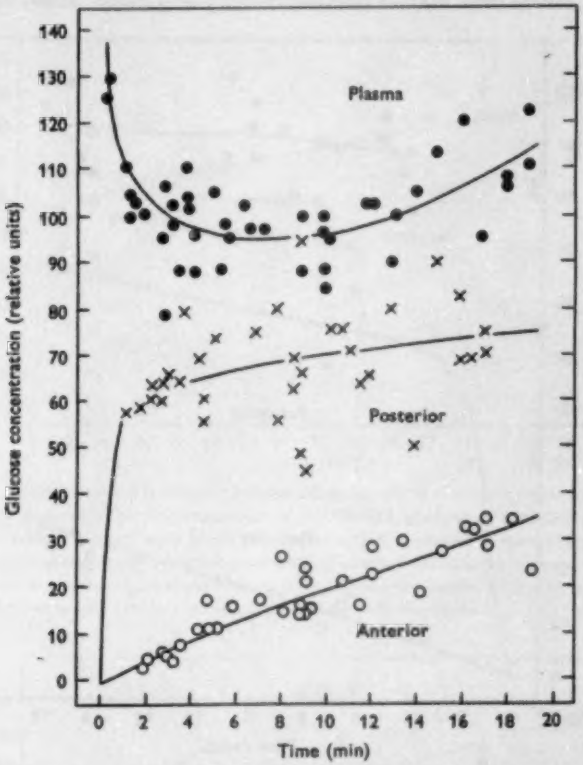

Fig. 3. Relative concentration of ^{14}C-labelled glucose in plasma and aqueous of anterior and posterior chambers in normal rabbits given insulin following parenteral administration.

DISCUSSION

While the principal purpose of this investigation was to study the effect of insulin on glucose transport into the intraocular chambers, which effect was found to be negligible, it is nevertheless interesting to observe that the absolute rate at which glucose enters the posterior chamber is extremely rapid. By means of mathematical methods of treating the data described elsewhere (Kinsey & Reddy, 1959), the turnover rate can be shown to be approximately 25%/min, a rate appreciably above that of several other

substances: Na 9%, Cl, 21% (Kinsey & Reddy, 1959), urea 9·6% (Kinsey, Reddy & Skrentny, 1960) and ascorbate 10% (Becker, 1959). Of the substances studied thus far, the turnover rate of glucose is exceeded only by that of bicarbonate. However, this substance is thought to enter not as an ion but as free carbon dioxide (Kinsey & Reddy, 1959). From the

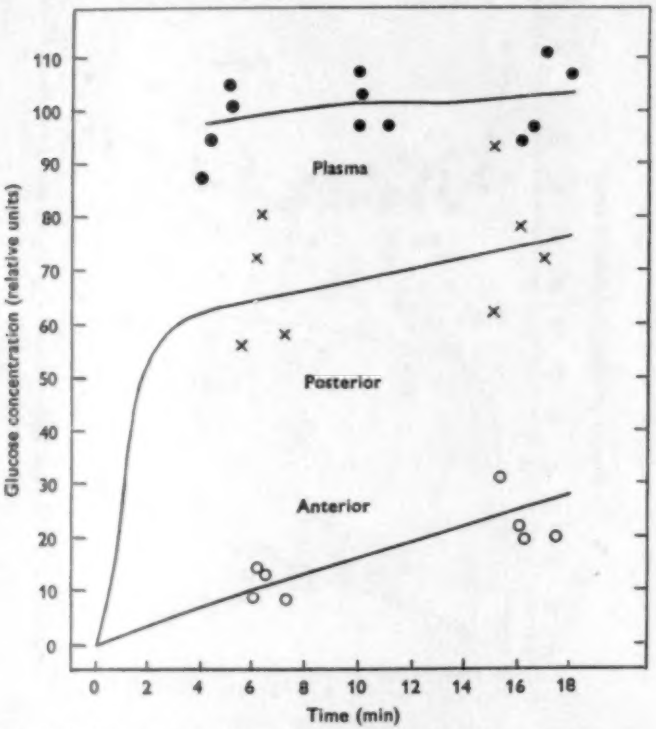

Fig. 4. Relative concentration of ^{14}C-labelled glucose in plasma and aqueous of anterior and posterior chambers in alloxan-injected animals in the hypoglycaemic phase of diabetes following parenteral administration.

rapidity of entrance of glucose and the fact that the steady-state ratio is less than one, it may be inferred that most of the glucose enters the posterior chamber by diffusion.

The turnover rate of glucose in the anterior chamber is similar to that of Na, i.e. 2·6%/min. Assuming a flow rate of 1·3%/min, it follows that since the concentration of glucose in the aqueous of the posterior chamber is less than that in plasma, slightly over half the glucose must enter the anterior chamber by diffusion.

Concerning the possible effect of insulin on glucose transport into the anterior chamber, the authors are unable to explain the differences between the results reported in the present study and those of Ross. In any case there is no evidence that insulin acts on the ciliary process to affect transport of glucose into the posterior chamber.

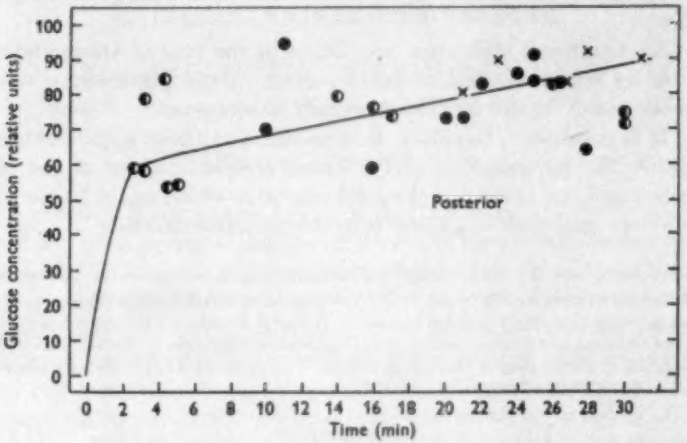

Fig. 5. Relative concentration of glucose with respect to plasma in the aqueous of posterior chamber; ◐, concentration of ^{14}C-labelled glucose in normal animals after elevation of blood sugar level; ●, concentration of glucose in normal animals, as determined chemically, following infusion of unlabelled sugar; ×, concentration of glucose in insulin-injected animals, as determined chemically, following infusion of unlabelled sugar; the line is a duplicate of the one shown in Fig. 1.

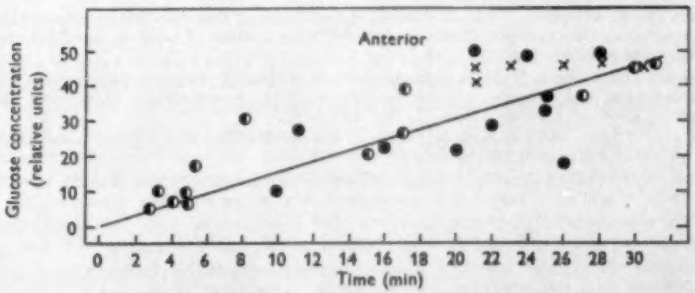

Fig. 6. Relative concentration of glucose with respect to plasma in the aqueous of anterior chamber; ◐, concentration of ^{14}C-labelled glucose in normal animals after elevation of blood sugar level; ●, concentration of glucose in normal animals, as determined chemically, following infusion of unlabelled sugar; ×, concentration of glucose in insulin-injected animals, as determined chemically, following infusion of unlabelled sugar; the line is a duplicate of the one shown in Fig. 1.

SUMMARY

1. The rate of accumulation of glucose in the aqueous humour of the posterior and anterior chambers was studied with ^{14}C-labelled and unlabelled glucose in normal rabbits, in alloxan-diabetic rabbits in both the hypo- and hyperglycaemic phases of the disease and in animals given exogenous insulin.

2. No significant difference was found in the rate of accumulation of glucose by either method of measurement in the posterior or anterior chamber of any of the groups of animals investigated.

3. It is concluded, therefore, that insulin is without appreciable effect on either the permeability of the blood–aqueous barrier of the ciliary processes and iris or on any chemical reactions which might be associated with active transport of glucose into the posterior chamber.

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THE INHIBITORY ACTION OF THE SYMPATHETIC NERVES ON THE SMOOTH MUSCLE OF THE RABBIT GUT, ITS REVERSAL BY RESERPINE AND RESTORATION BY CATECHOL AMINES AND BY DOPA

BY J. S. GILLESPIE AND B. R. MACKENNA
From the Department of Physiology, University of Glasgow

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In a previous investigation (Gillespie & Mackenna, 1960) rabbits were given daily intravenous injections of reserpine for several days and the responses of an innervated preparation of the colon from these animals were studied *in vitro*, the entire parasympathetic and sympathetic outflows being available for separate stimulation. It was expected that reserpine, which is believed to deplete adrenergic nerve endings of their chemical transmitter sympathin, would abolish the inhibitory effect of sympathetic nerve stimulation; in fact, inhibition was consistently replaced by a large contraction. This finding was the starting point of the investigations reported in this paper. In addition it has been found that in preparations from reserpine-treated rabbits the inhibitory effect of sympathetic nerve stimulation can be restored by soaking in adrenaline or noradrenaline; the mechanism of this restoration has been studied. A preliminary account of these results has already been published (Gillespie & Mackenna, 1959).

METHODS

Rabbits of either sex were killed by a blow on the neck and bled. Two types of innervated intestinal preparations were used. First, the doubly innervated preparation of the rabbit colon in which the extrinsic sympathetic (lumbar colonic) and parasympathetic (pelvic) nerves can be separately stimulated, by means of fluid electrodes. The dissection of this preparation and the details of the electrodes have been described (Garry & Gillespie, 1955). The second type of preparation consisted of a length of about 4 cm of mid ileum together with the mesentery and mesenteric artery supplying the region. The artery, plus some adjacent mesentery, was drawn into a fluid electrode and the periarterial nerves were stimulated. The preparations were suspended in a 200 ml. bath filled with Krebs's saline solution at 36° C and oxygenated by a gas mixture of 95 % O_2 and 5 % CO_2. The composition of the saline solution was (g/l.) NaCl 6·92, KCl 0·35, $CaCl_2$ 0·28, KH_2PO_4 0·16, $NaHCO_3$ 2·1, $MgSO_4.7H_2O$ 0·29, glucose 2·0. The contractions of the preparations were recorded with a light isotonic gimbal lever exerting a tension of 0·5 g and writing sideways on a smoked drum. The nerves were stimulated with 1 msec rectangular pulses whose voltage was adjusted to give maximal responses. The duration of stimulation was usually 15 sec and the frequency 10/sec for the pelvic nerve and 50/sec for the lumbar colonic nerves. Where other frequencies or durations of stimulation were used this is indicated.

In a number of rabbits, at a preliminary aseptic operation, the pelvic nerves were cut and allowed to degenerate for 14 days. A single mid-line skin incision over the sacrum and coccyx allowed access through the sciatic notch on each side to both pelvic nerves at their origin from the lumbosacral plexus. A Zeiss binocular dissecting microscope providing illumination along the optical axis was found to be of great assistance. Each pelvic nerve was tied before cutting and one end of the ligature was left long for later identification. A surface antibiotic, Polybactrin, was used on the various layers during closure of the wound. Mock operations were performed, identical in all respects with the single omission that the pelvic nerves after exposure were neither tied nor cut. Rabbits whose pelvic nerves were cut had difficulty in urinating, so that the bladder contents had to be expelled daily either by abdominal pressure or by catheterization.

Reserpine (Serpasil; Ciba) was given by daily intravenous injections for 1–10 days, and the animal was killed on the day following the last injection. The dose of reserpine varied according to the number of injections. The technique in most experiments was to give 5 injections; 0·2 mg/kg daily for the first 3 days, then 1 mg/kg daily for the final 2 days. Rabbits treated with reserpine were kept in a warm room at 29° C.

Several rabbits were given daily intravenous injections of appropriate volumes of the vehicle in which the reserpine was dissolved; these control solutions were provided by Messrs Ciba Ltd. The reserpine used for *in vitro* experiments was the dry powder dissolved in 10% ascorbic acid. Other drugs used in these experiments were acetylcholine chloride, hexamethonium bromide, atropine sulphate, 2:6 xylyl choline ether bromide (TM 10), ergotamine tartrate. Concentrations of these drugs refer to the salts, while concentrations of the following refer to the base: adrenaline hydrochloride, noradrenaline hydrochloride, dopamine, DOPA, L-tyrosine, and tolazoline (Priscol).

RESULTS

The action of reserpine in vivo

The response of the innervated colon preparation from a normal rabbit to stimulation of its extrinsic nerves is shown in Fig. 1*A*, and that of an innervated ileal preparation in Fig. 8. The periarterial nerves of the ileum and the sympathetic (lumbar colonic) nerves of the colon, when stimulated, cause inhibition whereas the parasympathetic (pelvic) nerves of the colon cause contraction. In preparations from rabbits injected with reserpine intravenously for 1–10 days previously, the response to sympathetic nerve stimulation is converted to contraction in both the colon and the ileum (Fig. 1*B* and *C*); the response of the colon to stimulation of the pelvic nerve is unaltered (Fig. 1*B*). The motor response to sympathetic nerve stimulation after reserpine never exceeds the contraction produced by pelvic nerve stimulation. It is not due to an altered response of the smooth muscle to the normal transmitter, since the response to noradrenaline and to adrenaline added to the bath remains inhibitory. In the colon there is a remarkable resemblance between the motor sympathetic response after reserpine and the pelvic response. This is not due to an escape of current from the stimulating electrodes on the sympathetic nerves to the pelvic parasympathetic fibres in the other electrode. Thus, ligation of the sympathetic nerves 1–2 mm distal to the fluid electrode abolishes the

sympathetic motor response without altering the contraction due to pelvic nerve stimulation, and the response can be obtained in preparations in which the pelvic nerves are not retained.

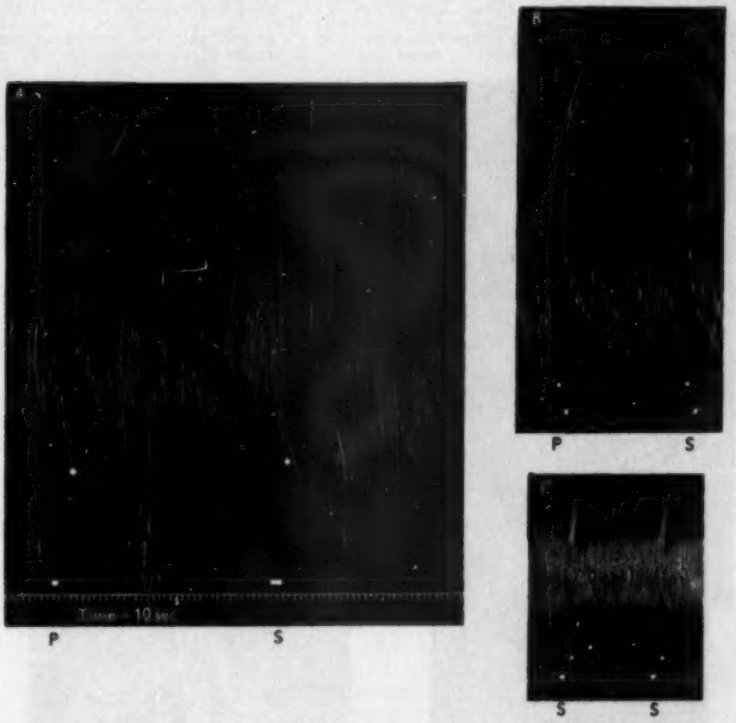

Fig. 1 *A*. The response of the colon from a normal rabbit to stimulation of the parasympathetic and sympathetic nerves. *B* and *C*, the response of the colon and of the ileum, respectively, from an animal treated with reserpine to stimulation of the parasympathetic (P) and sympathetic (S) nerves. After reserpine the response to sympathetic nerve stimulation is contraction. Time marker, *B* and *C*, 30 sec.

Effect of hexamethonium (C_6). This motor response to sympathetic nerve stimulation in an animal previously treated with reserpine is abolished by C_6 in concentrations similar to those required to block the response to stimulation of the preganglionic pelvic fibres. Furthermore, the rate of onset, the final degree of block, and the rate of recovery on washing are similar for both responses. An example of this blocking action of C_6 is shown in Fig. 2.

Effect of atropine. Atropine sulphate abolished the motor response to sympathetic nerve stimulation in concentrations similar to those required

to abolish the motor response to stimulation of the pelvic nerve. This is illustrated in Fig. 3. As with C_6, the rate of onset and degree of block were similar for the two responses.

Effect of frequency. From the effect of C_6 and atropine it seemed that the motor sympathetic response was due to stimulation of preganglionic

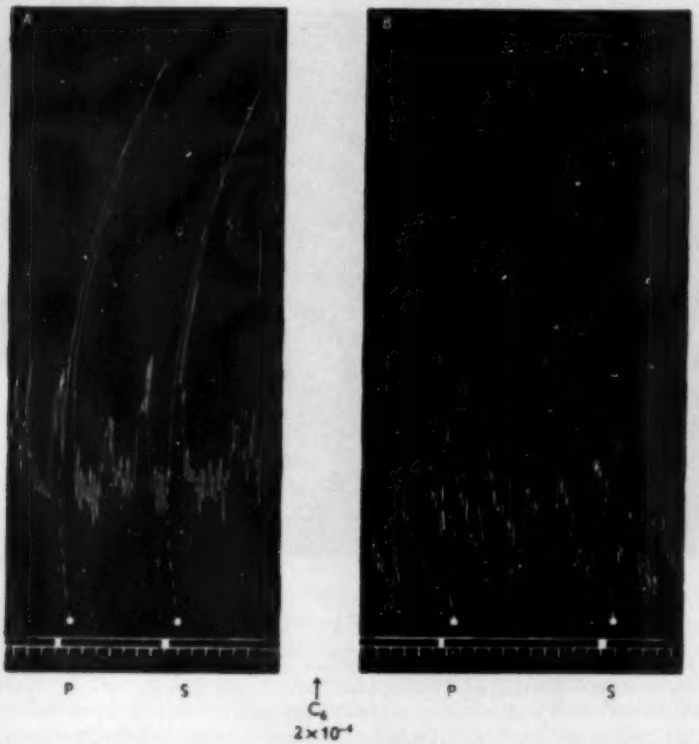

Fig. 2. Action of hexamethonium bromide on the motor response of the rabbit colon to sympathetic nerve stimulation; rabbit previously treated with reserpine. The motor responses to parasympathetic (P) and to sympathetic (S) nerve stimulation shown in *A* are blocked in *B* after hexamethonium bromide 2×10^{-4}. In this and in all subsequent figures, time marker, 30 sec.

cholinergic fibres similar to those in the pelvic nerve. The parasympathetic nature of these fibres in the sympathetic pathway was further suggested by the finding that the frequency sensitivity was similar to that of the fibres in the pelvic nerve and unlike that of the sympathetic inhibitory fibres. It is known from previous work that the threshold frequency for the parasympathetic response from the colon is less than 1/sec, and maximum

responses are produced at about 10/sec; the threshold frequency for the normal sympathetic inhibitory response is > 2/sec and the maximum response is not reached until about 50/sec (Garry & Gillespie, 1955). The motor response to sympathetic nerve stimulation after reserpine has a frequency sensitivity similar to that of the normal parasympathetic fibres, i.e. its maximum is reached at 10/sec. and its threshold is below 2·5/sec (Fig. 4).

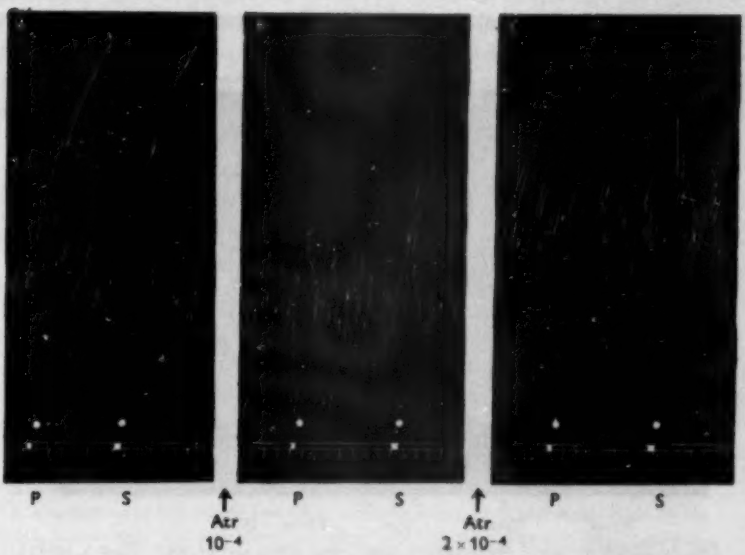

Fig. 3. Action of atropine on the sympathetic motor response of the colon from a rabbit previously treated with reserpine. Responses to stimulation of the sympathetic (S) and parasympathetic (P) nerves in *A* are partially blocked by atropine 10^{-4} in *B* and completely blocked by atropine 2×10^{-4} in *C*.

Origin of the motor fibres. The simplest explanation of the results so far described is that there are some few parasympathetic nerve fibres in the sympathetic nerves which we were stimulating. The effect of stimulating these parasympathetic fibres is normally masked by the much greater number of sympathetic inhibitory fibres. After inactivation of the sympathetic fibres by reserpine the motor effect is uncovered. In the periarterial nerves of the ileal preparation this is a likely explanation. Vagal fibres run in the mesentery and stimulation of the periarterial nerves can, even without reserpine, sometimes produce motor effects (Finkleman, 1930). The origin of the cholinergic fibres in the sympathetic nerves to the colon might be the pelvic nerves. To determine whether this is so, both

pelvic nerves were cut in a preliminary aseptic operation and allowed to degenerate for 14 days. In the last 5 days of this period the animal was given reserpine daily by intravenous injection. Innervated colon and ileal preparation were set up simultaneously in separate baths. The innervated ileal preparation served as a control and showed that reserpine had induced reversal of the normal sympathetic inhibitory response to contraction. In the colon preparation the cut peripheral ends of both pelvic nerves together with adjacent mesentery were pulled into one electrode. Stimulation of this tissue demonstrated whether degeneration was complete. It

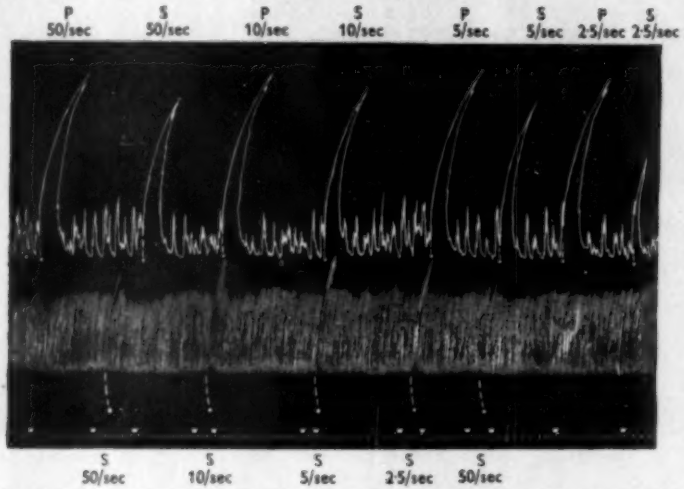

Fig. 4. Preparations of colon (upper trace) and ileum (lower trace) from a rabbit treated previously with reserpine. Stimulation of the parasympathetic (P) and sympathetic (S) nerves with different frequencies of stimulation shows that the motor response to sympathetic nerve stimulation can still be elicited at frequencies as low as 2·5/sec.

was found to be difficult to destroy all the parasympathetic outflow. A similar difficulty was experienced previously with the lumbar outflow (Gillespie & Mackenna, 1960).

The results of these experiments showed clearly that the motor response to sympathetic stimulation after administration of reserpine was dependent on an intact pelvic nerve pathway. When nerve degeneration was complete, as judged by the absence of a response to stimulation of the peripheral end of the cut pelvic nerve, then the motor response to sympathetic nerve stimulation was also abolished. When there was a small residual contraction on stimulation of the pelvic nerve, then there was a similar small contraction on stimulating the sympathetic nerves. The

motor response to sympathetic nerve stimulation never exceeded the response to pelvic nerve stimulation so that the normal relationship between the two was preserved.

Effect of pelvic nerve fatigue. If the motor response to sympathetic nerve stimulation be dependent on pelvic (parasympathetic) fibres, then fatigue of the pelvic nerves as well as degeneration might reduce or abolish the sympathetic motor response after reserpine. Fatigue was produced by stimulating the pelvic nerves continuously at a frequency of 50/sec for long periods. The results of one such experiment are shown in Fig. 5. When the pelvic nerve pathway is fatigued, as evidenced by the decline in the initial contraction, the motor response to sympathetic nerve stimulation is also abolished, and a small inhibitory effect may be unmasked.

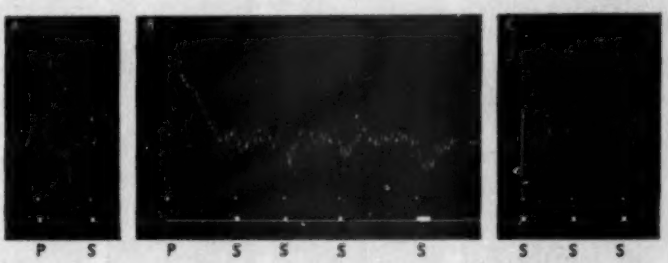

Fig. 5. Effect of fatigue of the parasympathetic nerve on the sympathetic motor response of the colon in a rabbit previously treated with reserpine. *A*, motor response to both parasympathetic (P) and sympathetic (S) nerve stimulation. In *B* stimulation of the parasympathetic nerve is begun at P and maintained to the end. Interpolation of four short periods of sympathetic nerve stimulation demonstrates the reappearance of an inhibitory effect. On recovery from pelvic nerve fatigue the motor effect of sympathetic nerve stimulation is restored (*C*).

Action of ergotamine, tolazoline and 2:6 *xylyl choline ether bromide* (*TM* 10). If the motor response to sympathetic nerve stimulation after reserpine were due to the presence of some motor fibres, then it should be possible in normal preparations to use other sympathetic blocking agents to convert the inhibition to contraction. Ergotamine was chosen because of the report by Hawkins & Paton (1958) that it blocked the inhibitory action of nicotine on the bronchial smooth muscle; tolazoline (Priscol), because of a report by Varagic (1956) that it successfully blocked the inhibitory response of the rabbit colon to sympathetic nerve stimulation; and TM 10, because it blocks the inhibitory effect of sympathetic nerve stimulation on the small and large intestine (Bain & Fielden, 1956; Gillespie & Mackenna, 1960).

None of these blocking agents converted the inhibitory response of the

colon to sympathetic nerve stimulation into a contraction. Of the three, only TM 10 produced a selective block of the sympathetic inhibitory effect, abolishing the inhibition from sympathetic nerve stimulation while leaving the motor effect of pelvic nerve stimulation unimpaired (Fig. 6). Neither ergotamine nor tolazoline proved to be satisfactory selective blocking agents for the present purpose. In the colon tolazoline in concentrations up to 10^{-5} commonly enhanced the inhibitory effect of sympathetic nerve stimulation; higher concentrations reduced inhibition but at the same time reduced as effectively the motor response to pelvic nerve stimulation. Ergotamine tartrate had little effect on the responses to parasympathetic

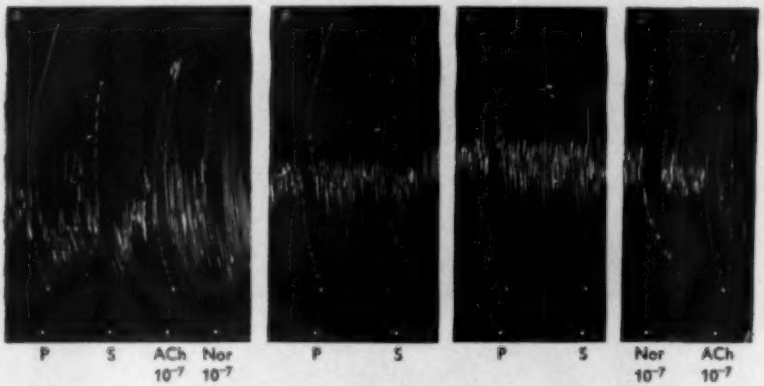

Fig. 6. Action of 2:6 xylyl choline ether bromide (TM 10) on the normal rabbit colon. *A*, responses to parasympathetic (P) and to sympathetic (S) nerve stimulation, and to acetylcholine (ACh) and noradrenaline (Nor). *B*, 20 min, and *C*, 70 min, after adding TM 10 (10^{-5}). *C*, sympathetic inhibition is abolished with only slight reduction in the parasympathetic motor response. *D*, response to Nor is enhanced; that to ACh unaltered.

or to sympathetic stimulation. In the ileum both tolazoline and ergotamine tartrate enhanced the inhibitory effect of periarterial nerve stimulation if frequencies of 50/sec and above were used; at lower frequencies, however, either pure motor or biphasic responses could be obtained after the addition of one of these drugs.

Restoration of inhibition by adrenaline or noradrenaline. At some point in each experiment on the colon or ileum from rabbits treated with reserpine, noradrenaline was added to the bath to show that the direct response of the smooth muscle to the adrenergic transmitter remained inhibitory. On occasions the concentration of noradrenaline was as high as 10^{-5}. It was sometimes noted, after noradrenaline was washed out and rhythmic activity and tone had returned to normal, that on stimulating

the sympathetic nerves their inhibitory effect was restored (Fig. 7). This restoration could not be demonstrated in every preparation; sometimes, although the motor response of the colon to sympathetic nerve stimulation was abolished after exposure to noradrenaline, there was no reappearance of inhibition. Adrenaline was as effective as noradrenaline in restoring the inhibitory effect.

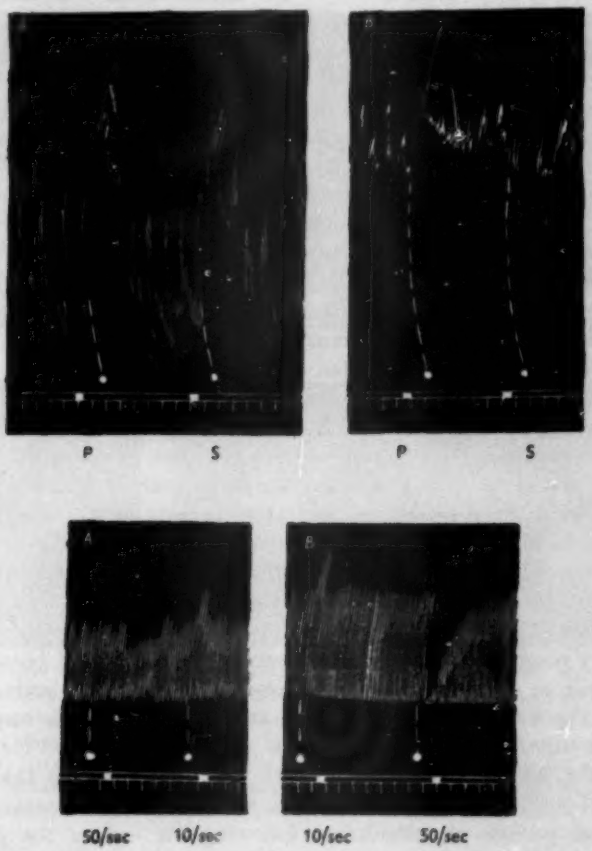

Fig. 7. Preparations of the colon (upper trace) and ileum (lower trace) from a rabbit previously treated with reserpine. Panels *A* show initial motor responses to sympathetic (S) and to parasympathetic (P) nerve stimulation. Between *A* and *B* noradrenaline (5×10^{-6}) added for 15 min and then removed. *B* shows the restoration of the inhibitory effect of sympathetic nerve stimulation. In the ileum restoration of inhibition was apparent only at a stimulation frequency of 50/sec.

The optimal concentration of noradrenaline for restoring the inhibitory response was investigated in the ileum. Four adjacent lengths of ileum, complete with mesenteric nerves, from an animal treated with reserpine, were suspended in separate baths and the motor responses of each to periarterial stimulation were demonstrated. The preparations were then exposed for a period of 30 min to noradrenaline in a concentration of either 5×10^{-7}, 5×10^{-6}, 10^{-5}, or 5×10^{-5}. The noradrenaline was then washed out and the responses to nerve stimulation were re-examined. Inhibition now occurred with all four concentrations; it was greater after 5×10^{-6} noradrenaline than after 5×10^{-7}, but concentrations greater than 5×10^{-6} were no more effective. The optimal time of exposure to noradrenaline was roughly determined in a similar way. Four adjacent lengths of ileum were exposed to a concentration of noradrenaline of 5×10^{-6} for either 5, 15, 30, or 60 min. The inhibitory effect of nerve stimulation was restored in each. Inhibition was greater in the preparation soaked for 15 min than in that soaked for only 5 min, but exposure for longer than 15 min did not enhance the effect.

The restored inhibitory effect of sympathetic nerve stimulation after soaking in adrenaline or noradrenaline was easily fatigued. This was best seen if the nerves were stimulated continuously at a frequency of 50/sec for 5 min. In a preparation of normal ileum sympathetic nerve stimulation causes complete inhibition which is maintained throughout the 5 min (Fig. 8, upper trace). Nevertheless, some decline in transmitter output probably does occur during such prolonged stimulation. This can be shown by comparing the responses to short test periods of stimulation before, and then at intervals after, the prolonged stimulation (Fig. 8*A*). After the prolonged stimulation the inhibitory effect of brief test periods of stimulation is diminished and recovers relatively slowly. By contrast, in similar preparations of ileum from reserpine-treated rabbits in which inhibitory responses to sympathetic nerve stimulation had been restored by soaking in noradrenaline, the prolonged stimulation leads to rapid fatigue (Fig. 8*A* and *B*). In panel *A* are shown repeatable responses to the short test stimuli. The preparation was then stimulated for 5 min at 50/sec (Fig. 8*B*). The inhibitory effect was short-lived, so that toward the end of the 5 min period there was no visible effect of nerve stimulation. Short test periods of stimulation following the end of the prolonged stimulation showed, however, that the fatigued nerves regained their inhibitory effect with a time course similar to that in the normal preparation. From these experiments it seems that the re-incorporated catechol amine is not all immediately available for release. Fatigue can occur with loss of any visible effect of nerve stimulation at a time when the transmitter is still present in the nerve endings.

Action of noradrenaline precursors. If noradrenaline precursors such as dopamine, DOPA and L-tyrosine could also restore the inhibitory response, then it might be argued that the synthetic pathways for the sympathetic transmitter were still functioning, especially since DOPA and tyrosine have no inhibitory effect of their own. Secondly, such experiments might provide information that the nerve endings were

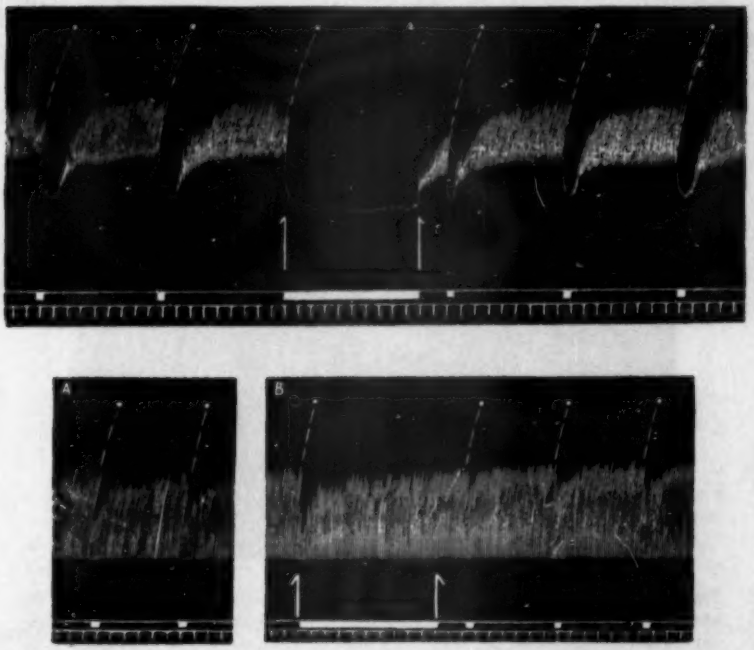

Fig. 8. Upper trace is from a preparation of ileum from a normal animal showing responses to periarterial nerve stimulation at 50/sec for short periods and for 5 min (between arrows). The inhibition is maintained throughout the 5 min period of stimulation. Lower traces, *A* and *B*, ileum preparation from a rabbit previously treated with reserpine. The inhibitory effect of periarterial nerve stimulation has been restored by soaking in noradrenaline but is readily fatigued during prolonged stimulation at 50/sec, recovering with rest. For details see text.

depleted not only of the final transmitter but also of some or all of its precursors.

Dopamine 10^{-5} had by itself only a slight inhibitory action on the rabbit intestine. It could, however, restore the inhibitory effect of nerve stimulation at least as well as adrenaline or noradrenaline (Fig. 9). Dopa 10^{-5} had itself no inhibitory effect on the colon, yet it also restored the inhibitory

effect of sympathetic nerve stimulation (Fig. 10). Tyrosine was difficult to use because of its poor solubility. In concentrations up to 10^{-5} it was ineffective in restoring the inhibitory action of the sympathetic nerves in either the colon or the ileum.

The action of reserpine in vitro

In all the experiments described so far the reserpine was administered by daily intravenous injection for some days before the experiment. When reserpine was added to the bath fluid in which a preparation from a normal

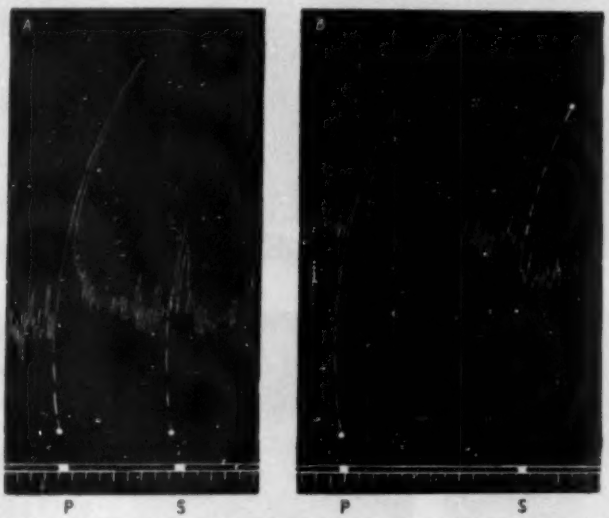

Fig. 9. Restoration of the inhibitory effect of sympathetic nerve stimulation (S) after soaking in dopamine. *A*, the responses of the rabbit colon to sympathetic (S) and to parasympathetic (P) nerve stimulation; the animal had previously been treated with reserpine. Between *A* and *B* the preparation was soaked in dopamine 10^{-4} for 1·5 hr. After the dopamine was washed out the response to sympathetic nerve stimulation was inhibitory.

animal was suspended, the effects could be divided into two groups, early and late. Only the late effects showed any specificity for the adrenergic nerves and corresponded to the effects seen from *in vivo* administration.

Almost immediately after adding reserpine to the bath fluid there was a gradual progressive loss of rhythmic activity and of tone. The rate of development was dependent on the concentration of reserpine. Accompanying this loss of rhythmic activity the ability of the preparation to respond to pelvic or lumbar nerve stimulation, to acetylcholine or to noradrenaline, was abolished or greatly reduced. These are the early effects

of reserpine. They are non-specific and affect equally the responses to stimulation of either nervous outflow and to the appropriate transmitters. The effects are reversible, and on washing out the reserpine the inhibitory response to sympathetic nerve stimulation is restored. The effect of reserpine at this stage of its action is reminiscent of that produced by anoxia.

If reserpine is left in the bath the late effects appear. After some time, usually 3–4 hr, the rhythmic activity begins again as intermittent groups of spike-like contractions. Coincident with this the motor response to stimulation of the pelvic nerve begins to improve and the first sign of a

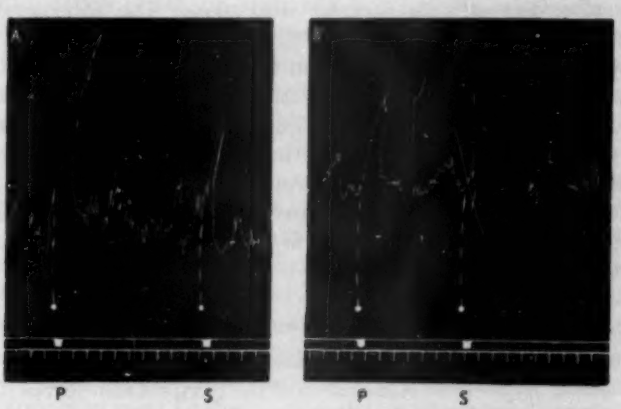

Fig. 10. Restoration of the sympathetic inhibitory effect in a colon from a reserpine-treated rabbit after soaking in DOPA. *A*, initial responses to stimulation of the parasympathetic (P) and sympathetic (S) nerves. Between *A* and *B* DOPA 10^{-4} left in contact for 1 hr. After washing, the response to sympathetic nerve stimulation is now inhibitory.

specific effect on the sympathetic nerves appears—stimulation now causes contraction. In spite of the partial recovery of the pelvic nerve response the smooth muscle shows no improvement in its response to acetylcholine. If now the reserpine is removed by washing, the rhythmic activity and tone return almost to their previous level, the contraction to pelvic nerve stimulation improves still further, acetylcholine and noradrenaline regain their ability to cause contraction and inhibition, respectively, but the response to sympathetic nerve stimulation remains motor and does not return to inhibition. This conversion of the response to sympathetic stimulation from inhibitory to motor persists for the remainder of the experiment. These late effects correspond to the effects produced by giving reserpine intravenously.

In these experiments solid reserpine was dissolved in 10% solution of ascorbic acid. Ascorbic acid left in contact with the preparation for 30 min

did not alter the response to stimulation of either pelvic or lumbar nerves or to noradrenaline or acetylcholine. The delayed action of the reserpine–ascorbic acid solution *in vitro*, so similar to the action of reserpine *in vivo*, was attributed to reserpine. The action of ascorbic acid alone, when left in contact with the preparation for several hours, was unfortunately not tested. Since then this experimental control has been done. In two out of three experiments on the colon the inhibitory response produced by sympathetic nerve stimulation was reversed to contraction and in three out of four experiments on the ileum the inhibitory response became biphasic after exposure to ascorbic acid alone. The concentration of ascorbic acid was 5×10^{-4} (1 ml. of 10 % solution added to a 200 ml. bath) and the time required for reversal about 4 hr. The reversal once established with ascorbic acid was as stable as with the reserpine and ascorbic acid mixture. These experiments make it impossible to attribute to reserpine itself any action *in vitro* in reversing the inhibition produced by sympathetic nerve stimulation. The action of reserpine *in vivo* is not affected by this finding, since we had shown that preparations from rabbits injected with the vehicle in which the reserpine was dissolved responded normally to stimulation of their nerves.

DISCUSSION

In a previous study of this preparation we were never able to demonstrate a motor response to stimulation of the sympathetic nerves (Garry & Gillespie, 1955). The very considerable motor response to sympathetic nerve stimulation which we have now found is clearly mediated by cholinergic fibres, as is evidenced by its remarkable similarity to the response to pelvic nerve stimulation and by its equal sensitivity to block by atropine and hexamethonium. Since the classical work of Dale & Feldberg (1934) on sudomotor nerves, the presence of cholinergic fibres in what are anatomically sympathetic nerves has been increasingly recognized. Burn & Rand (1960*b*) have suggested that the cholinergic fibres in the sympathetic nerves innervate 'stores' of noradrenaline in the tissues. Impulses in these cholinergic fibres normally lead to liberation of noradrenaline from the 'stores' and bring about an adrenergic response. Nicotine-like drugs can also liberate noradrenaline from these 'stores'. If the 'stores' are depleted by reserpine, then the adrenergic effect is lost and the liberated acetylcholine may diffuse away from the site of liberation around the 'stores' and reach other effectors, with the production of a cholinergic response. However, such an explanation cannot be applied to the present sympathetic motor response of the rabbit colon, since it is abolished by section and degeneration and by fatigue of the pelvic nerves, whose integrity is therefore essential to this response.

The suggestion that, after reserpine treatment, sympathetic nerve stimulation can somehow activate the parasympathetic pathway is so unorthodox that the possibility of experimental error or misinterpretation requires particularly careful consideration. The possibility that the pelvic nerve or its branches were stimulated by current escape can be excluded, since a ligature around the sympathetic nerves 1–2 mm distal to the fluid electrode abolishes the motor effect previously obtained from these nerves without affecting the contraction produced by pelvic nerve stimulation. Moreover, when TM 10 was used to block the inhibition from sympathetic nerve stimulation it did not uncover a sympathetic motor response; yet the motor effect from pelvic nerve stimulation was still elicitable and would have been recorded in the event of current spread to the pelvic nerves or their branches. This result with TM 10 is also inconsistent with a second possibility, that a few fibres of pelvic-nerve origin are present among the sympathetic fibres of the lumbar colonic nerves. Langley & Anderson (1896) showed by degenerative experiments that the pelvic fibres leave the hypogastric nerve in the sacral colonic nerves and do not ascend as far as the inferior mesenteric ganglia. In agreement with this, Garry & Gillespie (1955) found that cutting the hypogastric nerves just below their origin from the inferior mesenteric ganglia did not reduce the motor response to stimulation of the pelvic nerve, indicating that few, if any, motor fibres ascend the hypogastric nerve to join the lumbar colonic nerve. By contrast, the motor response to sympathetic nerve stimulation is often almost as great as that to stimulation of the pelvic nerve and has as short a latency (Figs. 1*B*, 2, 3, 4), which does not seem consistent with the concept of a few fibres being responsible. Finally, the possibility that the nerves are in fact a mixture of sympathetic and parasympathetic fibres was specifically investigated in a previous paper (Garry & Gillespie, 1955). It was shown then that if such a mixture were present then it should be possible to demonstrate the presence of the two groups of fibres by varying the frequency of stimulation. Such a mixture was never found. In the absence of a ready explanation we should like to raise the possibility that in the gut, after treatment with reserpine, sympathetic nerve stimulation can, in some way unknown, activate the parasympathetic fibres.

Reserpine produces an apparently specific depletion of 5-hydroxytryptamine and catechol amines. These amines are found intracellularly in special sites and in high concentration. Reserpine interferes with the mechanism maintaining this high intracellular concentration so that the amines 'leak' through the membrane and are lost. The site of action of reserpine in producing this effect may be intracellular on some binding sites for the amines or on some carrier system at the cell membrane which maintains the concentration difference. Whatever the site of action,

however, the cells should lose, after reserpine, their ability to concentrate and retain these amines. From the present experiments, and those of Burn & Rand (1960*a*), it appears, on the contrary, that after reserpine the nerve endings are still able to take up catechol amines from the medium surrounding them and to retain them for at least some hours. From the effectiveness of extreme dilutions (5×10^{-7}) of noradrenaline it is probable that the nerve endings are also still capable of concentrating the amines.

Since DOPA and dopamine are able to restore the response, whereas tyrosine is not, we should like to suggest that the rate-limiting step in normal synthesis is the conversion of tyrosine to DOPA and that this synthesis is slow. Thus a small increase in the rate of amine 'leak' produced by reserpine will, if it exceeds maximum synthesis, lead ultimately to depletion of the transmitter and all precursors subsequent to tyrosine. Evidence that normal synthesis of catechol amines in the adrenal gland is limited by slow conversion of tyrosine to DOPA has been presented by Udenfriend & Wyngaarden (1956). Our suggestion, that the loss of amine stores and of nerve function is finally due to the normal rate of synthesis being inadequate to compensate for a small 'leak' of transmitter due to reserpine, receives support from investigations on the output of catechols from rabbit adrenals (Kroneberg & Schümann, 1958). If the depletion of transmitter were due solely to its liberation, then one might expect removal of this stored product to result in an acceleration of its synthesis. The output of amine after reserpine should show a large initial increase due to the liberation of the stores; following exhaustion of these stores, the output should fall but remain at a level higher than normal because of the postulated acceleration in synthesis. In fact the output of adrenaline in the adrenal venous blood is raised only during the first few hours following reserpine administration (Kroneberg & Schümann, 1958).

In those experiments in which reserpine was added to the bath fluid the effects mimicked exactly those resulting from intravenous administration of the drug. Unfortunately interpretation is impossible because of the finding that the vehicle, ascorbic acid solution, could itself, on occasion, produce similar effects. It is interesting, however, that Eliasson, von Euler & Stjärne (1955) have demonstrated the release of noradrenaline from the spleen by ascorbic acid in concentrations producing only a small change in pH (0·3). It is possible that ascorbic acid also acts by liberating transmitter at a rate exceeding that at which it can be replaced by synthesis.

SUMMARY

1. The effect of reserpine on the responses *in vitro* of two types of innervated preparations of the rabbit intestine have been studied: (i) the rabbit colon, when stimulating extrinsic sympathetic and parasympathetic

nerves; (ii) preparations of the mid ileum, when stimulating the periarterial nerves.

2. After pre-treatment with reserpine by intravenous injection for 1–10 days, the response to sympathetic or periarterial nerve stimulation was converted from inhibition to contraction. The response to parasympathetic nerve stimulation and to noradrenaline or adrenaline was unaltered. Injection of control animals with appropriate quantities of the vehicle in which the reserpine was dissolved had no effect on the nerve response.

3. After reserpine treatment the motor responses from parasympathetic and from sympathetic nerve stimulation were similar in appearance. The contraction to sympathetic nerve stimulation never exceeded that due to parasympathetic nerve stimulation. Both responses were abolished by atropine and by hexamethonium in similar concentrations. Evidence is given that the sympathetic motor response was not due to current escape stimulating the parasympathetic nerve.

4. Three sympathetic blocking agents, ergotamine tartrate, tolazoline (Priscol) and 2:6 xylyl choline ether bromide (TM 10), failed to uncover a motor response to sympathetic nerve stimulation in normal preparations. Only TM 10 produced a satisfactory selective sympathetic block.

5. The motor response to sympathetic nerve stimulation is abolished after section and degeneration of the pelvic nerves or after stimulation of these nerves to exhaustion.

6. From these observations it is concluded that the motor response to sympathetic nerve stimulation after reserpine is mediated by the parasympathetic nerves and is not due to cholinergic fibres in the sympathetic outflow.

7. The inhibitory effect of the sympathetic nerves could be restored by soaking the preparation in a solution of either adrenaline or noradrenaline (optimal concentration 5×10^{-6}) and subsequent washing. It is suggested that restoration is due to re-incorporation of transmitter into the nerve endings. Dopamine and DOPA were also effective, probably by serving as substrates for transmitter synthesis; L-tyrosine was ineffective. The effectiveness of DOPA and the ineffectiveness of L-tyrosine suggest that the synthesis of DOPA from tyrosine is slow. It is suggested that the final depletion of transmitter by reserpine is due to a small 'leak' of transmitter which is greater than the maximum rate of re-synthesis.

8. The inhibitory effect restored by re-incorporation of amines was easily fatigued by continuous stimulation of the nerves. On stopping stimulation recovery occurred, suggesting that all the restored transmitter was not equally available for release.

9. Reserpine dissolved in ascorbic acid and added to the bath fluid of

in vitro preparations from normal animals appeared to produce an identical reversal of the inhibitory effect of sympathetic nerve stimulation. Ascorbic acid itself was found, subsequently, to be able to produce a similar reversal.

We are indebted to Messrs Ciba Ltd for generous supplies of Serpasil and of control solutions and to Miss A. Gilroy and Miss G. Docherty for careful technical assistance. The Rankin Medical Research Fund helped to provide apparatus.

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THE INFLUENCE OF BROMIDE IONS ON EXCITATION–CONTRACTION COUPLING IN FROG'S SKELETAL MUSCLE

BY G. B. FRANK

From the Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Canada

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The substitution of bromide for chloride ions in the solution bathing a skeletal muscle results in a marked increase in the twitch tension produced by a single supramaximal stimulus but is without effect on the maximum tetanic tension of the same muscle (Kahn & Sandow, 1955). This property is shared by other anions of the lyotropic series below chloride in their ability to salt out proteins from solution, notably nitrate, iodide, and thiocyanate (Kahn & Sandow, 1950, 1955; Lubin, 1957). The speed of onset of this effect of anion substitution in intact muscles (Kahn & Sandow, 1950; Hill & Macpherson, 1954) and in single, isolated muscle fibres (Horowicz & Hodgkin, 1956) has led to the conclusion that it is due to an action of the anions at the surface of the muscle fibres. It has been shown that the increase in the twitch tension is due to an increased duration of the active state of the contractile mechanism within the muscle fibres (Hill & Macpherson, 1954; Ritchie, 1954). Intracellular micro-electrode studies have shown that replacing chloride with these anions results in an increase in the amplitude and duration of the negative after-potential (Etzensperger & Bretonneau, 1956; Lubin, 1957). However, this increased depolarization does not seem sufficient to account for the increase in the mechanical response, and it has been suggested that the increased mechanical response results from some other action of these anions on the cell membrane (Lubin, 1957; Shanes, 1958).

Evidence has recently been presented supporting the hypothesis of Heilbrunn & Wiercinski (1947) and Sandow (1952) that an increased influx of calcium ions during depolarization may represent at least one step of excitation–contraction coupling (Frank, 1958, 1960). The present study was undertaken to investigate the possibility that the effects of anion substitution on contraction might be due to an effect of the anions on the calcium entry mechanism. This concept is supported by the results of the experiments presented below.

METHODS

The extensor longus digiti IV muscle of the frog *Rana pipiens* was used in all experiments, which were performed at room temperature and at all seasons of the year.

Isometric responses were recorded with the muscle mounted vertically in a bath containing the appropriate solution. The lower end of the muscle was fixed and the upper end was attached to the free end of a lever above the bath by means of a nylon thread. Strain gauges were mounted on the lever near its fixed end and responses were recorded with a Grass Model 5 polygraph. For further details of the preparation see Frank (1960).

For electrical recording the muscle was mounted horizontally in a two-compartment bath (Frank, 1958). The compartments were isolated by means of a petroleum jelly gap 8 mm in length. Potential changes were induced by changing the composition of the solution in one of the compartments.

The primary solution had the following composition (mM): choline chloride, 111·8; KCl, 2·47; $CaCl_2$, 1·08; $NaHCO_3$, 2·38; NaH_2PO_4, 0·087; glucose, 11·1. Except for the absence of the $CaCl_2$, the composition of the calcium-free solution was identical. The isotonic potassium chloride solution contained 123 mM-KCl with or without the addition of 1·08 mM-$CaCl_2$. The 25 mM potassium solution was made by the addition of solid KCl to the primary solution. All other solutions with elevated potassium concentrations were made by mixing appropriate amounts of the primary and isotonic potassium chloride solutions. The bromide solutions were identical in composition, with choline bromide substituted for choline chloride and potassium bromide substituted for potassium chloride. Caffeine (Kahlbaum) was dissolved in a small quantity of solution with the aid of a few drops of concentrated HCl and diluted to the desired concentration. The resulting caffeine solution was buffered to pH 7·2 by the addition of solid $NaHCO_3$. In some of the experiments D-tubocurarine 10^{-4} g/ml. was added to the solutions or a gas mixture of 0·5 % CO_2 in O_2 bubbled through the solutions. Neither of these procedures modified the results obtained. Details of the precautions taken in preparing the water for the solutions, the selection of reagents and in other ways preventing contamination with calcium from any source have been described elsewhere (Frank, 1960).

The details of the general procedure used in most of the experiments were described previously (Frank, 1960). Briefly, this consisted of determining the response of the muscle to an elevated potassium concentration before and after treating the muscle with a solution of modified composition. Following each test with elevated potassium the muscle was kept in a solution containing 2·47 mM potassium for at least 10 min before another test was performed. Before each test with an altered solution a control response of the muscle was obtained.

RESULTS

Effects of substituting bromide for chloride ions on potassium-induced contractures

In studies using a single muscle fibre preparation, Horowicz & Hodgkin (1956) noted that substitution of nitrate for chloride ions increased the tension induced by low concentrations of potassium but had little effect on tensions induced by high concentrations of potassium. A similar effect is produced by substituting bromide for chloride ions, as is shown in Fig. 1*A* and *B*. In this experiment the maximum tension of the contracture produced by 25 mM potassium is about 4 times larger with the toe muscle in bromide solutions than it is with the muscle in chloride solutions. This observation has been repeated in some twenty muscles in which bromide

substitution increased the maximum tension from 4 to 12 times. As in the case of the effect of bromide substitution on the muscle twitch, the onset is rapid. The maximum increase in the contracture tension is generally obtained during the 10–15 sec needed for the contracture to reach its maximum. However, after the muscle had remained in the bromide solution for 30 min or more, up to 5 min was required for the potentiation to disappear after replacement of the bromide with chloride solution.

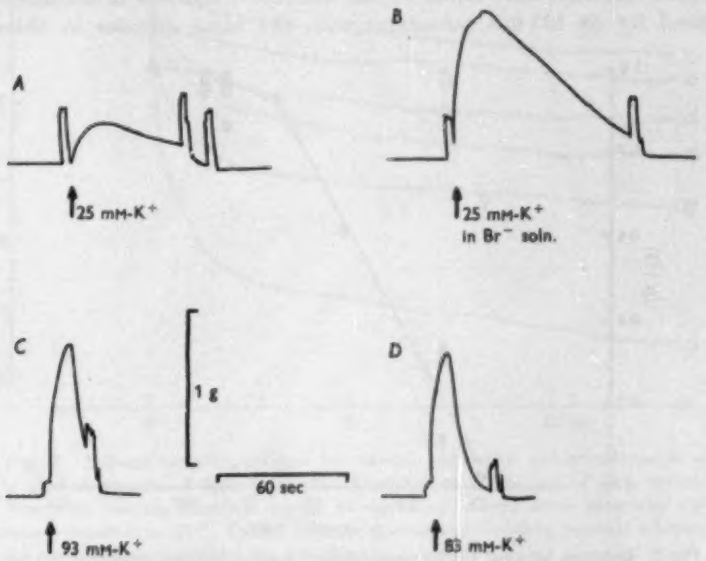

Fig. 1. Potassium-induced contractures in a single toe muscle of a frog. Responses *A*, *C*, and *D* obtained with the muscle in a chloride solution and *B* with the muscle in a bromide solution. The arrows indicate the points at which the test solutions were placed in the bath. The rectangular artifacts at the start and end of the contractures were caused by emptying the bath to change the solution.

Larger contracture tensions are obtained by increasing the potassium concentration above 25 mM. Thus it was possible to determine the potassium concentration in chloride solutions needed to cause a contracture equal to that due to 25 mM potassium in bromide solutions. This was done in the experiment shown in Fig. 1. In this case the maximum tension of the contracture produced by 25 mM potassium in bromide solution was between the maximum tensions produced by 83 and 93 mM potassium in chloride solution. Although contractures having similar maximum tensions can be obtained in this manner, it should be noted that the durations of the contractures produced by high potassium concentrations in chloride

(Fig. 1*C* and *D*) are considerably shorter than equal tension contractures induced by lower concentrations of potassium in bromide solutions (Fig. 1*B*).

The maximum tensions of contractures induced by various potassium concentrations in chloride and in bromide solutions are plotted in Fig. 2. The data obtained in two separate experiments are presented. The maximum tensions of 25 mM potassium-induced contractures with the muscles in bromide solutions were equal to the maximum tensions of contractures induced by 63–123 mM potassium with the same muscles in chloride

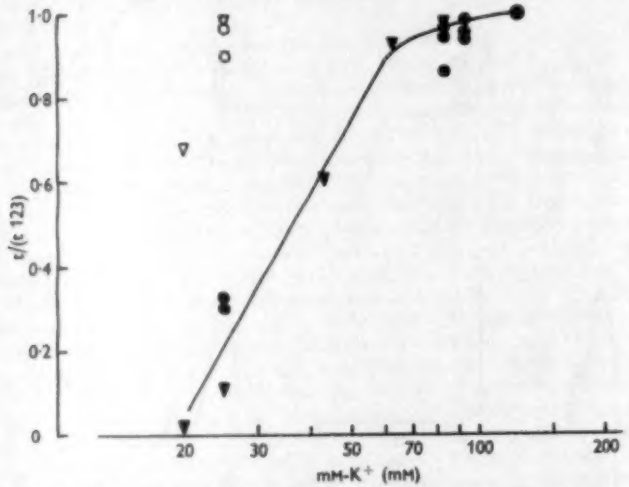

Fig. 2. Relation between the potassium concentration and the maximum tension of the contracture. Two experiments with different muscles, represented by different symbols. Open symbols, muscles in bromide solutions; solid symbols, muscles in chloride solutions. t/(t 123), maximum tension/maximum tension of 123 mM potassium-induced contracture. Semi-log. scale.

solutions. The relation between the potassium concentration and the maximum tension of the contractures produced in chloride solutions was similar in other muscles in which the effect of bromide was not determined. The only difference was that with some of these other muscles the curve was shifted to the right.

It was previously found that the maximum tensions of contractures produced by isotonic potassium chloride solutions in the small toe muscles used in these experiments were equal or nearly equal to the maximum tetanic tensions (Frank, 1960). It was found in the present study that bromide substitution had little or no effect on the maximum tension or the duration of contractures produced by 123 mM potassium solutions.

This seems to indicate that the events in excitation–contraction coupling are the same in the tetanus and in the maximum contracture.

When recordings were made at the time of substituting bromide solutions for chloride, a small contracture was observed. This contracture was never more than a quarter the size of contractures induced by 25 mM potassium.

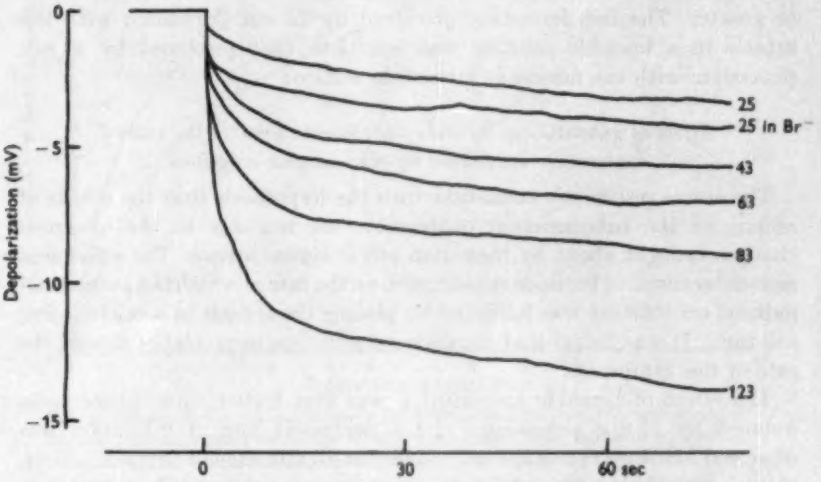

Fig. 3. Depolarizations produced by various potassium concentrations in a single toe muscle. Potassium concentrations given at the end of each record. Muscle in chloride solutions during all recordings except while recording the record marked 'in Br^-'. In this case the muscle was kept in a bromide solution for 5 min before and during the recording.

Effects of substituting bromide for chloride ions on potassium-induced depolarizations

Hutter & Padsha (1959) reported that substitution of nitrate for chloride ions increased the amplitude of the end-plate potential in a nerve–muscle preparation and of the electrotonic potentials in isolated frog muscle. These effects, plus the frequently reported increase in the size of the negative after-potential, made it desirable to study the effects of bromide ions on potassium-induced depolarizations.

When a bromide solution replaced the chloride solution bathing the part of a muscle in one of the compartments of the bath, that part of the muscle was depolarized about 25% as much as by 25 mM potassium. This depolarization might well be responsible for the slight contracture which resulted from bromide substitution for chloride ions.

As can be seen in Fig. 3, the depolarization produced by 25 mM potassium

is slightly larger when the muscle is in a bromide solution. However, this slight increase seems inadequate to account for the large increase in the size of the contracture (Figs. 1 and 2). In order to produce contractures in chloride solutions having maximum tensions comparable to those produced by 25 mM potassium with the muscle in a bromide solution, it was necessary to use solutions having potassium concentrations of 63 mM or greater. The depolarization produced by 25 mM potassium with the muscle in a bromide solution was less than that produced by 43 mM potassium with the muscle in a chloride solution.

Effect of substituting bromide for chloride ions on the rate of contracture inhibition by calcium-free solutions

The above results are consistent with the hypothesis that the effects of anions on the submaximum contracture are not due to the electrical changes brought about by these non-physiological anions. The effect was next determined of bromide substitution on the rate at which the potassium-induced contracture was inhibited by placing the muscle in a calcium-free solution. It was found that bromide substitution appreciably slowed the rate of this inhibition.

The effect of bromide substitution was first tested with contractures induced by 25 mM potassium, and a decreased rate of inhibition was observed. However, two factors reduce the significance of this test. First, the contractures produced by 25 mM potassium are considerably larger when the muscle is kept in bromide solutions than when it is in a chloride solution. Secondly, an increase in the maximum tension of 25 mM potassium-induced contractures generally is observed if the response is tested shortly after exposing the muscle to a calcium-free solution (Frank, 1960). This increase is not observed in bromide solutions. Consequently, the inhibition curves obtained in the two types of solutions have distinctly different shapes. For these reasons the rest of the experiments were performed using 123 mM potassium solutions to induce contractures.

As has been shown above, the maximum tensions of contractures produced by isotonic potassium chloride and by isotonic potassium bromide were equal, or nearly so. However, the contractures were inhibited more slowly in calcium-free bromide solutions. The results of a typical experiment were plotted in Fig. 4. Experiments of this type were carried out with eleven different muscles. In each case a definite decrease in the rate of inhibition was found. Unfortunately, owing to instrument difficulties, quantitatively reliable results were obtained in only three of these experiments. The length of time in the calcium-free solutions required to produce a 50% decrease in the maximum tensions of the contractures is shown in Table 1.

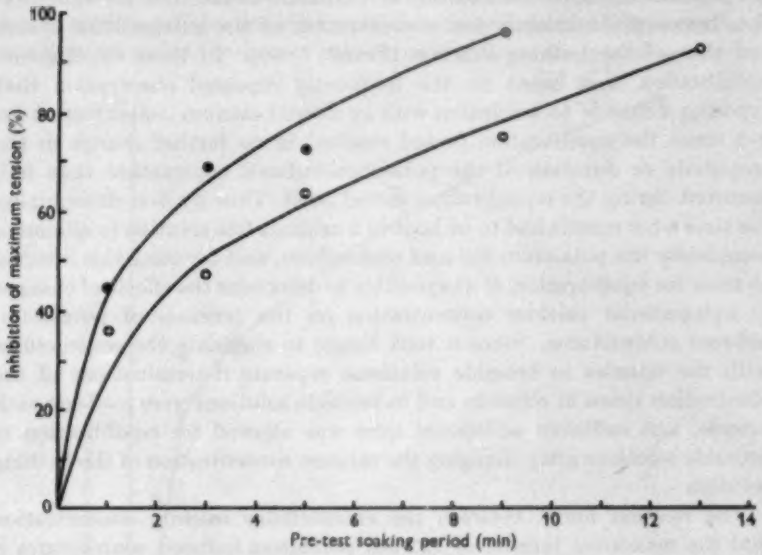

Fig. 4. Effect of substituting bromide for chloride solutions on the rate at which 123 mM potassium-induced contractures of a toe muscle were inhibited by placing the muscle in a calcium-free solution. Abscissa, time muscle was kept in a calcium-free solution before testing with potassium. ●, muscle in chloride solutions; ○, muscle in bromide solutions. Lines drawn by eye.

Table 1. Time in the calcium-free solution required to produce a 50 % decrease in the maximum tensions of 123 mM potassium-induced contractures

Muscle	In chloride (min)	In bromide (min)
1113	1·4	2·6
1209	2·0	7·0
1215	1·5	2·7

In three additional experiments only the time in a calcium-free solution required to eliminate completely the contracture was determined. In each of these cases the exposure time for complete elimination of the mechanical response was at least 50 % longer with the muscle in a bromide solution.

Effects of changes in extracellular calcium concentration on the maximum tensions of contractures produced by isotonic potassium chloride and isotonic potassium bromide

It was previously shown that the length of time for which a toe muscle has to be kept in a calcium-free solution in order completely to eliminate

the potassium-induced contracture is a measure of the time for equilibration between the calcium ion concentration of the extracellular spaces and that of the bathing solution (Frank, 1960). In these experiments equilibration was based on the frequently repeated observation that exposing a muscle to a solution with an altered calcium concentration for 2–3 times the equilibration period resulted in no further change in the amplitude or duration of the potassium-induced contracture than had occurred during the equilibration period itself. Thus by first determining the time a toe muscle had to be kept in a calcium-free solution to eliminate completely the potassium-induced contracture, and by using this interval to allow for equilibration, it was possible to determine the effects of changes in extracellular calcium concentration on the tensions of potassium-induced contractures. Since it took longer to eliminate the contractures with the muscles in bromide solutions, separate determinations of the elimination times in chloride and in bromide solutions were made on each muscle, and sufficient additional time was allowed for equilibration in bromide solutions after changing the calcium concentration of the bathing solution.

The relation found between the extracellular calcium concentration and the maximum tension of 123 mM potassium-induced contractures is shown in Fig. 5. This type of relation was previously described for 25 mM potassium-induced contracture in chloride solutions (Frank, 1960). The curve obtained with 123 mM potassium-induced contractures does not show the initial potentiation as the calcium concentration is lowered as previously noted with 25 mM potassium-induced contractures.

In Fig. 5 the results are presented for three experiments in which the relation between extracellular calcium and contracture tension was determined with the muscles in chloride and in bromide solutions. It can be seen that substitution of bromide for chloride ions did not change the relation between the extracellular calcium concentration and the maximum tension of 123 mM potassium-induced contractures. It was also observed that bromide substitution did not modify the duration of the contractures in this type of experiment.

Effect of substituting bromide for chloride ions on caffeine-induced contractures

It has been shown that essentially unmodified caffeine-induced contractures of skeletal muscle can be produced while the muscle is completely depolarized by potassium (Axelsson & Thesleff, 1958), or in the complete absence of extracellular calcium (Frank, 1960). It would seem that caffeine causes a mechanical response either by a process different from depolarization-induced contractures or by an action on the same excitation–contraction process but at a stage which follows depolarization and calcium

influx. Therefore it was of interest to see whether bromide substitution would modify caffeine-induced contractures and particularly whether it would increase the tensions of submaximal caffeine contractures.

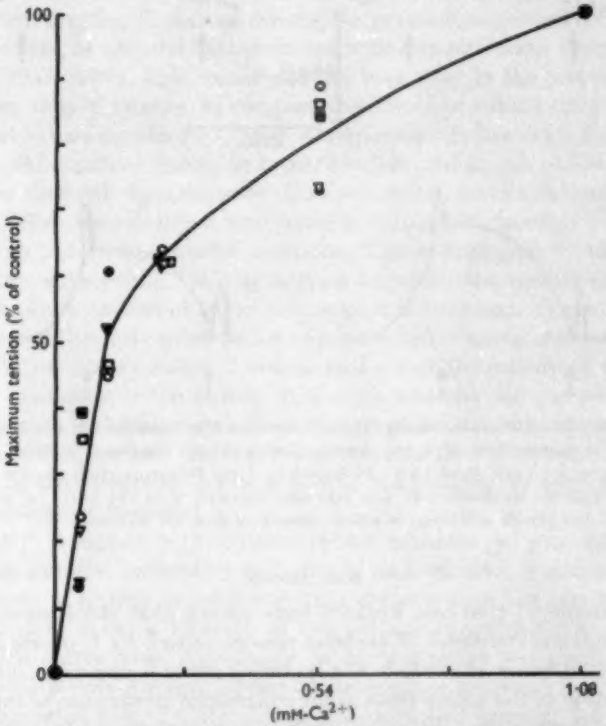

Fig. 5. Relation between the extracellular calcium concentration and the maximum tension of 123 mM potassium-induced contractures of the toe muscle. Experiments with three different muscles represented by different symbols. The open symbols represent determinations made with the muscles in chloride solutions and the solid symbols determinations with the muscles in bromide solutions.

Caffeine contractures of one muscle produced in chloride and in bromide solutions are shown in Fig. 6. The maximum tension of the contracture produced by caffeine $5{\cdot}0 \times 10^{-4}$ g/ml. (Fig. 6*C*) was about the same as that produced by isotonic potassium chloride in the same muscle. In four experiments of this type the only change observed following bromide substitution was a slight but definite decrease in the maximum tensions of the caffeine-induced contractures.

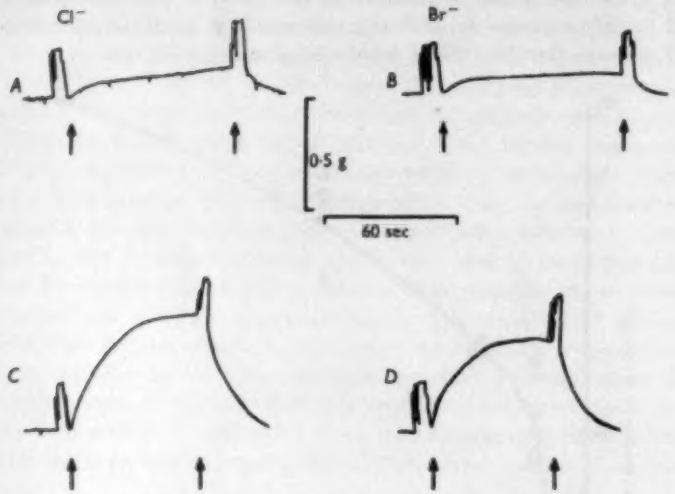

Fig. 6. Effect of substituting bromide for chloride solutions on the caffeine-induced contractures of a toe muscle. Test solutions contained caffeine $2{\cdot}5 \times 10^{-4}$ g/ml. in *A* and *B*, and $5{\cdot}0 \times 10^{-4}$ g/ml. in *C* and *D*. Arrows indicate the times when caffeine solutions were put into and removed from the bath. *A* and *C*, muscle in chloride solutions; *B* and *D*, muscle in bromide solutions.

DISCUSSION

The results of previous workers have shown that the increase in the size of maximal twitches of skeletal muscle caused by bromide ions and other anions below chloride in the lyotropic series is due to an increase in the duration of the active state of the contractile mechanism of the muscle cells, and further that the action of these ions is at the surface of the cells and not directly on the contractile elements within the cells (see p. 35). Therefore the effect of these anions on contraction results either from an increased depolarization during an action potential, or from an action on the processes which link the electrical and mechanical events, or from both.

At present excitation–contraction coupling can be presumed to occur in stages, starting with depolarization of the muscle fibre and ending with activation of the contractile mechanism. The substitution of bromide for chloride ions can increase the tension of twitches and of some types of submaximum contractures. However, the effects of bromide substitution on the depolarizations seem inadequate to account for the effects on the mechanical responses. Lubin (1957) recognized that the increase in the amplitude and duration of the negative after-potential of skeletal muscle fibres was not sufficient to account for the increase in the maximal twitch

which results from substitution of these anions for chloride. However, he suggested that the same change at the muscle fibre surface must be the cause of both effects.

In the previously reported studies from this laboratory on excitation–contraction coupling in skeletal muscle, the potassium-induced contracture has been used as a model for the events which occur during a contraction (Frank, 1958, 1960). This model also has been used in the present study. Therefore, it is of interest to compare the effects of substituting bromide for chloride ions on the two types of responses. It has been found that bromide substitution results in a marked increase in submaximum contractures induced by relatively low potassium concentrations but is without effect on maximum contractures induced by isotonic potassium chloride or potassium bromide solutions. This is analogous to the effects of bromide substitution on contractions in which the maximum twitch tension but not the maximum tetanic tension is increased. The rapid onset of the effect of bromide substitution on submaximum contractures indicates that, as in the muscle twitch, bromide ions act at the surface of the cells. Another similarity is the finding of a slight but obvious increase in the depolarization which accompanies each type of mechanical response in the presence of bromide ions. As is probably the case for the twitch, this slight increase is insufficient to account for the increase in the tension of the submaximum contracture.

Recently Hodgkin & Horowicz (1960) reported a potentiation of potassium-induced contractures in single skeletal muscle fibres by the substitution of nitrate or thiocyanite for chloride ions. As reported here they observed a slight increase in the potassium depolarization in the presence of nitrate ions. This change in depolarization was insufficient to account completely for the increase in contracture tension.

It is suggested here that the effects of substituting bromide for chloride ions on the mechanical responses of skeletal muscles are mainly due to an action of these anions on the calcium entry mechanism which links electrical and mechanical events in contraction. The evidence supporting the calcium entry mechanism of excitation–contraction coupling can be found elsewhere (see Frank, 1960, for references). The demonstration of a direct effect of bromide substitution for chloride ions on the rate at which the potassium-induced contracture is inhibited when the toe muscle is placed in a calcium-free solution supports the mechanism proposed here for the effects of bromide ions. Furthermore, the maximum possible tension of a potassium-induced contracture is determined by the extracellular calcium-ion concentration, and the inability of bromide ions to increase the tension of contractures made submaximum by reduction of the extracellular calcium concentration (Fig. 5) indicates that the effects of bromide

substitution on the potassium-induced contracture also are dependent upon some action of calcium ions.

It has been shown previously (Frank, 1960) that the rate at which the potassium-induced contracture of the toe muscle is inhibited when the muscle is placed in calcium-free solution is determined by the rate at which calcium ions are removed from the extracellular spaces of the muscle. Since the presence of bromide ions does not change the relation between the extracellular calcium concentration and the maximum tension of the 123 mM potassium-induced contracture, the decrease in the rate of inhibition must mean that the presence of bromide ions has in some way slowed down the loss of calcium ions from some site in the muscle. Since it is hard to visualize how bromide ions could interfere with the diffusion of calcium ions out of the extracellular spaces of the muscle, it appears probable that bromide ions act on the muscle fibre membrane to slow the loss of calcium associated with these membranes when the calcium concentration of the surrounding fluid is reduced.

Shanes (1958) has suggested that the effects of these anions which increase the muscle twitch could be explained by assuming that they increase the calcium concentration in the muscle fibre membrane. Since substitution of these anions for chloride does not increase the maximum tetanic tension it must be assumed that the maximum possible tension of the muscle is set by the state of the contractile mechanism of the cells and thus is not increased by the increased influx of calcium ions associated with each action potential during a tetanus. However, this explanation cannot account for the inability of bromide ions to increase the tension of contractures made submaximal by reduction of the extracellular calcium concentration (Fig. 5). All that can be said is that in some way bromide substitution favours the influx of calcium ions during a depolarization, but this effect probably is not due simply to an increase in membrane calcium concentration.

Bromide ions increase the size of the muscle twitch by increasing the duration of the active state of the contractile mechanism (Hill & Macpherson, 1954; Ritchie, 1954). Sandow (1955) presented some records indicating that potassium-induced contractures could be increased in duration by using nitrate in place of chloride ions. However, an interpretation of these records is complicated by the fact that relatively large strips of sartorius muscle (at least 1 mm across) were used and by the fact that the test solutions contained only 100 mM potassium. In the present study maximum contractures produced by 123 mM potassium were not increased either in amplitude or duration by bromide substitution, and even contractures induced by 25 mM potassium, which were greatly increased in amplitude, were not noticeably increased in duration. The present results

can best be interpreted as showing an increased effectiveness of relatively small depolarizations in producing a mechanical response in the presence of bromide ions. If bromide ions have the same mechanism of action in modifying muscle twitches and contractures, the increased duration of the active state during the twitch response in the presence of bromide ions would be due to an increased ability of the negative after-potential to maintain the active state. Further, if as suggested here the effects of bromide substitution are due to an action on the calcium entry mechanism, there should be an increased influx of calcium ions during a twitch produced with the muscle in a solution containing bromide ions compared to that produced in chloride-containing solutions. Indeed, Bianchi & Shanes (1959) have shown an increased influx of calcium ions per twitch when nitrate ions were substituted for chloride ions in the solution bathing the sartorius muscle of the frog.

SUMMARY

1. It was observed that substitution of bromide for chloride ions in solutions bathing the extensor longus digiti IV muscle of the frog increased from 4 to 12 times the maximum tensions of contractures induced by 25 mM potassium, but was without effect on maximum contractures induced by 123 mM potassium. The depolarization produced by 25 mM potassium also was slightly increased but this increase seemed insufficient to account for the marked potentiation of the mechanical response.

2. Substitution of bromide for chloride ions decreased the rate at which 25 and 123 mM potassium-induced contractures are inhibited when the toe muscle is placed in a calcium-free solution.

3. Bromide substitution did not increase the size of 123 mM induced contractures made submaximum by decreasing the extracellular calcium concentration, nor did it increase the tension of submaximum caffeine-induced contractures.

4. It is suggested that the increase in twitch tension and in the tension of submaximum potassium-induced contractures which occur when bromide is substituted for chloride is due to an increase in the excitation of the contractile mechanism of the muscle cells by small depolarizations. Further, it is suggested that this increase in the effect of small depolarizations is due to a relatively greater influx of calcium ions during depolarization in the presence of bromide ions.

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A COMPARISON OF THE CIRCULATORY EFFECTS OF ANGIOTENSIN, VASOPRESSIN AND ADRENALINE IN THE ANAESTHETIZED CAT

By GWENDA R. BARER

From the Nuffield Institute for Medical Research, University of Oxford

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The chemical structures of angiotensin and vasopressin have been determined in recent years and both have been synthesized. Both are peptides of very high potency whose role in the body is incompletely understood. Angiotensin exists naturally in two forms, a decapeptide and an octapeptide which is derived from it and is believed to be responsible for the effects of angiotensin. Horse angiotensins differ from ox angiotensins in that the fifth amino acid is isoleucine instead of valine. The decapeptides and the horse (isoleucine) octapeptide but not the ox (valine) octapeptide have been isolated from natural sources. Both octapeptides have been synthesized (Rittel, Iselin, Kappeler, Riniker & Schwyzer, 1957).

Two forms of vasopressin have been isolated and synthesized; lysine vasopressin which is found in the hog, and arginine vasopressin which has been found in all other species examined (Bartlett, Jöhl, Roeske, Stedman, Stewart Ward & du Vigneaud, 1956).

In the present work a comparison has been made of the three pressor substances, adrenaline, synthetic angiotensin and synthetic vasopressin and their actions on cardiac output, peripheral resistance and regional blood flow have been investigated. A preliminary report of this work has been published previously (Barer, 1959).

METHODS

Cats (1·7–4·2 kg) were anaesthetized with ethyl chloride and ether and then given chloralose (60 mg/kg I.V.). Heparin (Boots Pure Drug Co., 10 mg/kg I.V.) was given after the completion of the dissection. A Starling Ideal pump was used for artificial respiration when the chest was opened.

Blood pressure was recorded by means of a capacitance manometer and Cambridge fast pen recorder (response of whole system flat $\pm 5\%$ to 25 c/s) or Evershed and Vignoles Duplex quick-response recorder (response flat $\pm 5\%$ to 20 c/s) or, occasionally, a mercury manometer. External jugular venous pressure and atrial pressures were measured with saline manometers.

Blood flow was measured with an electromagnetic flowmeter designed by Wyatt (1959, 1961*a*, *b*), which has a low resistance to blood flow and which was placed in continuity with

either arteries or veins. The pressure drop across the system depended mainly on the connecting tube and cannulae, since the flowmeter head consisted of a tube 3·3 cm long with a minimum diameter of 0·5 cm. In a typical experiment with the apparatus used for cannulating the main pulmonary artery the connecting tubes, cannulae and flowmeter head produced a drop in pressure of 3 mm Hg for a rate of flow of 316 ml./min (saline). Similarly in a circuit used for the mesenteric vein there was a drop in pressure of 1 mm Hg for a rate of flow of 50 ml./min and in another circuit used in the renal vein (the narrowest) there was a drop in pressure of 1·4 mm Hg for a rate of flow of 21 ml./min. The magnet was operated at the mains supply frequency of 50 c/s and the mean flow was continuously recorded. Alternatively, the phasic flow within the range 0–20 c/s was continuously recorded, the accuracy being limited by the introduction of the connecting tubes. The instrument recorded both forward and backward flow with linearity of $\pm 1\%$ and neither the base line nor sensitivity drift exceeded 1% of full scale per hour. Full scale represented either 1000 or 200 ml./min, depending on the setting of the range switch.

In a few experiments a bubble flowmeter or a density flowmeter (Dawes, Mott & Vane, 1953) was used. The right heart output was measured by the electromagnetic flowmeter placed in continuity with the main pulmonary artery; the method of cannulation was described by Barer & Nüsser (1957). The right heart output is less than the left heart output by the amount of blood which circulates from the bronchial artery to the pulmonary veins. The systemic vascular resistance was calculated as

$$\frac{\text{arterial pressure} - \text{venous pressure}}{\text{right heart output}}.$$

Details of the measurement of flow in different regions will be described with the results.

Hormones. The formula of the synthetic val-5 octapeptide *angiotensin* (Ciba, Basel) used was

H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe.OH.

The angiotensin was dissolved in sterile distilled water (20 μg/ml.) in a plastic container and kept in the refrigerator. No loss of potency was noticed over many weeks.

The synthetic lysine *vasopressin* was in a solution stated to contain 6 pressor units/ml. This concentrated solution and a tenfold dilution (600 m-u./ml.) in sterile normal saline were kept in a refrigerator, but slight deterioration may have taken place during the course of the work. A further tenfold dilution was made up during experiments for injecting small quantities. The formula was

$$\overline{\text{Cys-Tyr-Phe-GluNH}_2\text{-AspNH}_2\text{-Cys}}\text{-Pro-Hys-GlyNH}_2.$$

Adrenaline (British Drug Houses) was also used.

Injections. Intravenous injections were made into a small plastic cannula (capacity 0·02 ml.) inserted into the femoral vein, or into the rubber tubing of a larger glass cannula in the external jugular vein where they were washed in with 2 ml. normal saline. It was important to use the small cannula for injections when measuring cardiac output, since it was shown that a rapid intravenous injection of 2 ml. saline caused an increase in right heart output of about 6% lasting 30 sec. Intra-arterial injections were made into the tubing of the flowmeter circuit which was placed in continuity with an artery.

RESULTS

Blood pressure changes

Angiotensin. Figures 3*A*, 4*A* and 7*A* illustrate typical changes in blood pressure which followed intravenous injections of angiotensin (tested in 59 cats in doses of 0·02–5 μg). There was a rise in blood pressure followed

(usually but not always) by a slight fall and a secondary rise lasting for several minutes. Large doses increased the duration as well as the magnitude of the response. The pulse pressure was increased, the heart rate decreased and no tachyphylaxis was observed. It was not possible to make a valid comparison of the potency of angiotensin and adrenaline because the time course and nature of their actions were different. However, the high activity of the synthetic angiotensin was demonstrated by the observation that a dose of adrenaline 5–10 times greater than angiotensin (weight for weight) was required to produce a similar peak rise in blood pressure. On a molecular basis the ratio was still higher.

Vasopressin. Vasopressin (24–1000 m-u. intravenously in twenty cats) caused a small but frequently prolonged rise in blood pressure (Figs. 3*C*, 4*C*). Above the threshold dose, increasing doses produced increasing rises in blood pressure up to a point, after which further increases in dose produced no further increments in blood pressure. The maximum rise was usually 10–20 mm Hg, never more than 30 mm Hg. The pulse pressure was usually reduced and the heart rate decreased.

Adrenaline. Under the conditions of these experiments there was most commonly a biphasic blood pressure response to intravenous injections of adrenaline (tested in 50 cats in doses of 0·5–30 μg) as shown in Fig. 1*B*. Often the secondary fall in blood pressure was profound and the subsequent rise was to a mean level lower than the initial value. A monophasic rise in blood pressure was also observed (Figs. 4*B*, 7*C*). The pulse pressure was increased and the heart rate changes were variable.

Cardiac output

Angiotensin. Angiotensin (0·02–5 μg) was injected intravenously into eighteen cats while the right heart output was being measured. In fourteen cats a rise of blood pressure was often recorded without any change in cardiac output (Fig. 1*A*), but large doses sometimes caused either a rise or a fall in cardiac output. (In five of these cats doses of 1–4 μg caused a fall in output often accompanied by cardiac irregularities and in five others doses of 0·4–4 μg led to a rise in output.) In two cats there was always a rise in output and in two other cats only a fall in cardiac output was recorded (but one had a high pulmonary arterial pressure, and in the other slight pulmonary valve incompetence was suspected). When a rise in output was observed it sometimes took place early (four cats) but at other times (three cats) it began only during the secondary fall in blood pressure and continued during the secondary rise. Thus there was no constant change in cardiac output accompanying the rise in blood pressure caused by angiotensin, which is therefore mainly attributed to increased peripheral resistance (Fig. 1*A*).

The venous pressure was unchanged after small doses but sometimes rose slightly (0·5–1 mm Hg) after large doses.

Vasopressin. Vasopressin was injected into five cats in doses from 120–300 m-u. intravenously; smaller doses did not raise the blood pressure. There was no constant change in cardiac output and the rise in blood pressure is therefore attributed to increased peripheral resistance.

Adrenaline. In seventeen cats the cardiac output was increased by adrenaline (1–30 μg I.V.) except on the very few occasions when a large dose gave rise to irregularities of rhythm, when there was sometimes a fall in output. The output rose as the pressure rose, usually continued to rise during

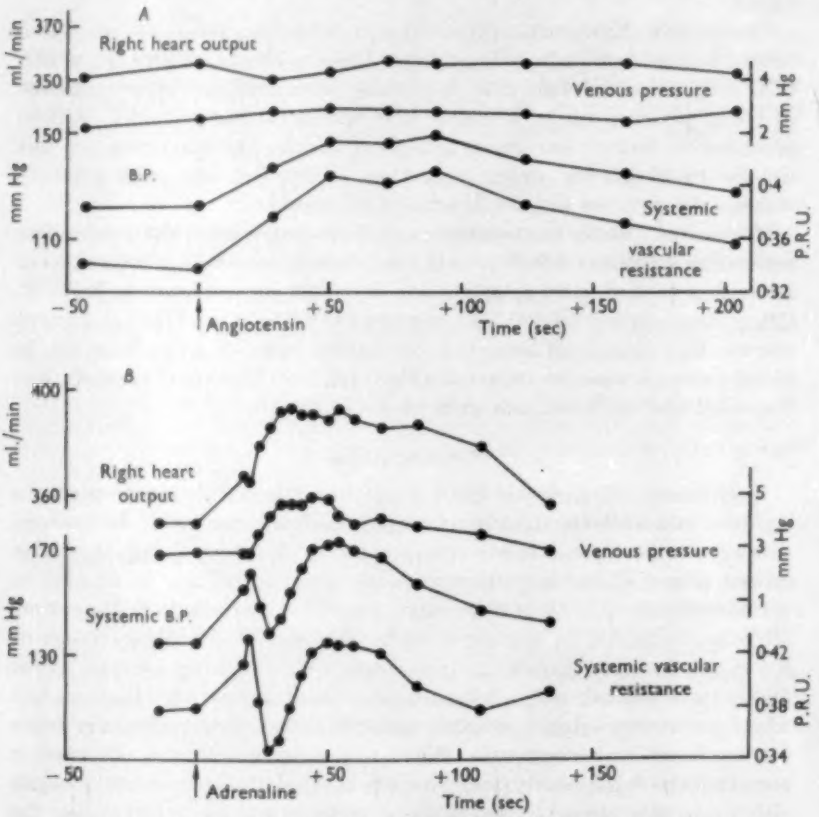

Fig. 1. 3·0 kg cat. The effect of angiotensin and adrenaline on right heart output and systemic vascular resistance (output measured with an electromagnetic flowmeter). *A*, injection of angiotensin 0·5 μg I.V. at arrow; *B*, injection of adrenaline 10 μg I.V. at arrow.

the subsequent fall in pressure, reached a maximum during the secondary rise in pressure, and remained up for several minutes (Fig. 1*B*). Thus the increase in output had a different time course from the blood pressure changes and continued whether the second phase of the blood pressure response was a rise or fall above the initial level. Venous pressure rose slightly (0·5–2 mm Hg). The calculated systemic vascular resistance usually rose, fell and rose again (Fig. 1*B*). Sometimes during a sustained rise in blood pressure the systemic vascular resistance remained at or below the original level.

Renal blood flow

The renal blood flow was measured by placing a flowmeter in the left renal vein (which was tied at its inferior vena caval end) and connecting it to the right external jugular vein. This procedure caused less disturbance than cannulating the two ends of the cut renal vein, because the external jugular vein was cannulated first and a single cannula was inserted rapidly into the renal vein, to minimize the interruption of renal blood flow. Renal arterial blood flow was measured by connecting the aortic end of the left carotid artery, through a flowmeter, to one of the left renal arteries.

Angiotensin. The effect of angiotensin on renal blood flow was investigated in fifteen cats (venous flow in eleven cats, arterial flow in four cats). The

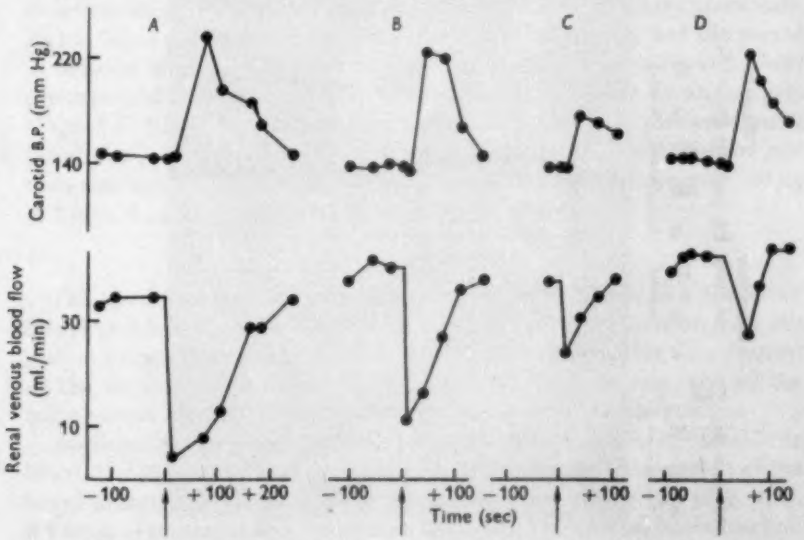

Fig. 2. 3·1 kg cat. The effect of angiotensin and adrenaline on renal venous blood flow (bubble flowmeter). *A*, angiotensin 4 μg I.V.; *B*, angiotensin 2 μg I.V.; *C*, angiotensin 1 μg I.V.; *D*, adrenaline 20 μg I.V.

rate of flow was invariably reduced by both intravenous and intra-arterial injections in proportion to the dose (Figs. 2, 3*A*. Doses were 0·1–4 μg I.V. in fifteen cats and 0·2–1·2 μg into the renal artery in two cats). The time course of the reduction in flow was usually similar to that of the blood-pressure changes but sometimes rather shorter. In two cats renal blood flow remained reduced during intravenous infusions of angiotensin (2 μg in 2 min, and 2 μg in 2½ min).

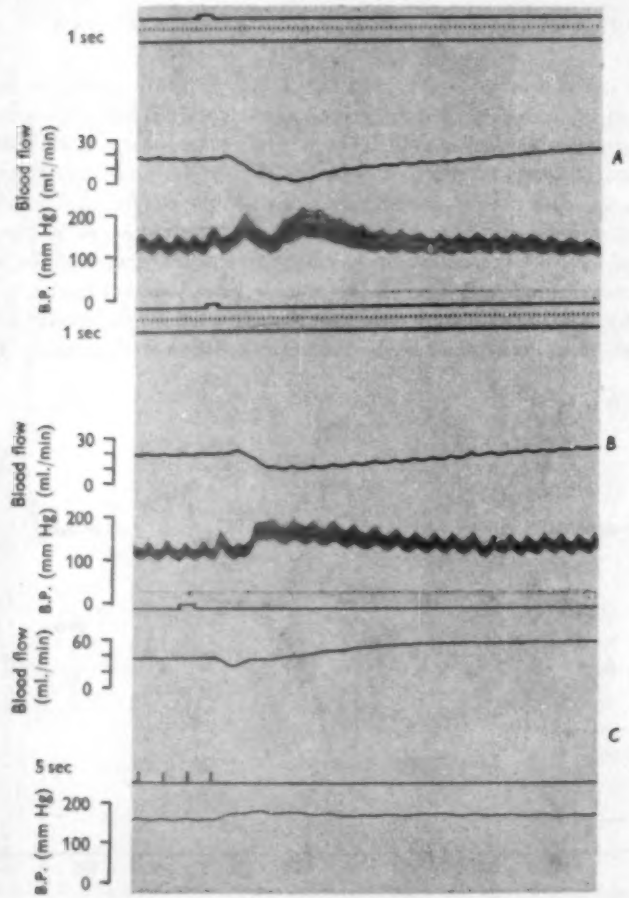

Fig. 3. Renal blood flow (electromagnetic flowmeter in a left renal vein). *A*, 2·9 kg cat, angiotensin 2 μg I.V. at signal; *B*, 2·9 kg cat, adrenaline 10 μg I.V. at signal; *C*, 3·2 kg cat, vasopressin 300 m-u. I.V. at signal.

Vasopressin. Vasopressin was investigated in twelve cats and was found to increase renal blood flow (measured in a vein in 8 cats and in an artery in four cats). In ten out of twelve cats given intravenous injections of vasopressin (24–300 m-u.) there were increases in blood flow. These increases were often (especially with large doses) preceded by a brief but large decrease in flow; the rise in flow was gradual, was often as great as 70% and lasted several minutes (Fig. 3*C*). Increases in renal blood flow were sometimes caused by doses which had no effect on the blood pressure (e.g. in one cat 60 m-u. caused a rise in flow of 23% without any change of blood pressure) and the increases in flow frequently outlasted the increases in blood pressure. In the two other cats which were given intravenous injections of vasopressin there was no change in blood pressure or renal blood flow even after large doses of vasopressin. Vasopressin was injected into the renal artery in four cats. In two of these the renal arterial blood flow was consistently increased by 60–240 m-u. of vasopressin and small doses had no effect on the blood pressure (Fig. 6*A*). In the third cat there was an increase in renal blood flow after three doses (80, 150 and 300 m-u.) but not after other similar doses. The fourth cat was one of the two already mentioned which were insensitive to all doses of vasopressin.

Adrenaline. In twelve cats adrenaline always reduced renal blood flow (venous flow was measured in ten cats and arterial flow in two cats; the doses were 0·5–20 μg I.V. in twelve cats and 5 μg into the renal artery in one cat). As the blood pressure rose the renal blood flow fell sharply but the period of reduced blood flow was usually shorter than that of increased blood pressure and relatively shorter than the decrease caused by angiotensin (Figs. 2*D*, 3*B*). There followed in most cats a period of increased renal blood flow. During infusions of adrenaline the renal flow decreased and then rose again while the blood pressure remained raised (three cats; 10 μg in 2 min, 6 μg in 4½ min, and 20 μg in 2 min 40 sec).

Mesenteric blood flow

The blood flow in the intestines was measured by means of a flowmeter introduced into the main mesenteric vein just before its junction with the gastric veins. Retrograde injection of dye showed that this vein drained all the small intestine except the first part of the duodenum, and all the colon except the final straight part which descends into the rectum.

Angiotensin. Angiotensin caused a profound reduction in mesenteric blood flow (0·2–4 μg I.V. in ten cats) whose time course followed that of the blood pressure except that its duration was often rather less (Fig. 4*A*). A period of increased flow sometimes followed. Intraportal injections had a very much smaller effect than injections into the external jugular vein on blood pressure and mesenteric blood flow, but whether this was due to

loss of angiotensin through diffusion in the liver or to inactivation could not be determined. After intravenous injections of angiotensin the portal venous pressure was constant or fell slightly during the period of reduced mesenteric blood flow but rose later (Fig. 5*A*). Injections into the central end of the flowmeter circuit (intraportally) led to a rapid rise in portal venous pressure but the systemic venous pressure remained unchanged.

Vasopressin. Vasopressin was tested in three cats (50–1000 m-u.) and in each of them caused a slow but prolonged decrease in mesenteric blood

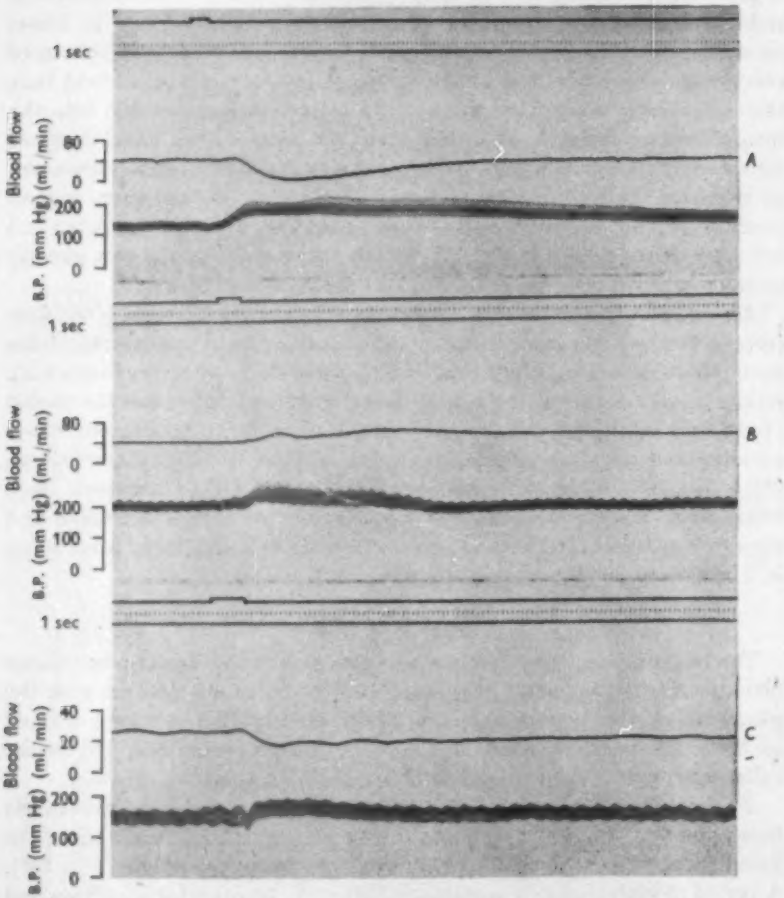

Fig. 4. Mesenteric venous blood flow (electromagnetic flowmeter). *A*, 3·3 kg cat, angiotensin 2 μg I.V. at signal; *B*, 2·3 kg cat, adrenaline 2·5 μg I.V. at signal; *C*, 1·9 kg cat, vasopressin 150 m-u. at signal.

flow (Fig. 4*C*). Recovery was incomplete and the effect of repeated doses is shown in Fig. 6*B*.

Adrenaline. The changes in mesenteric blood flow after intravenous injections of adrenaline (2·5–20 μg in eight cats) were variable. There was usually an increase in flow (Fig. 4*B*), but this was sometimes preceded or interrupted by a brief decrease. There was a large increase in portal venous pressure (Fig. 5*B*). After intraportal injections of adrenaline the changes in blood pressure and mesenteric blood flow were much diminished as compared with the changes after injections into the external jugular vein and there was a large rise in portal venous pressure.

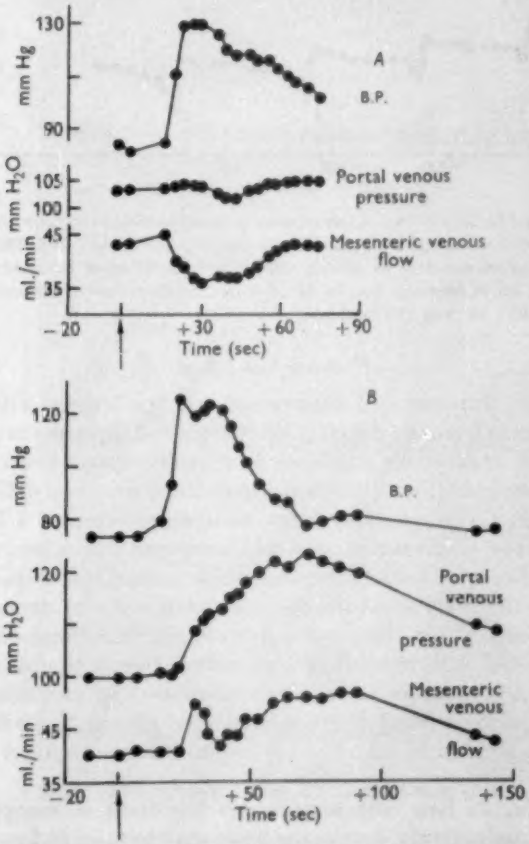

Fig. 5. 3·1 kg cat. The effect of angiotensin and adrenaline on portal venous pressure and mesenteric venous blood flow (electromagnetic flowmeter). *A*, angiotensin 2 μg I.V.; *B*, adrenaline 20 μg I.V.

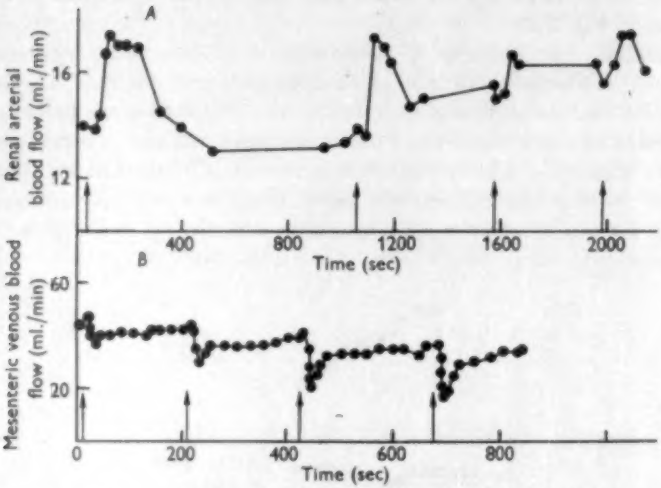

Fig. 6. *A*, 2·7 kg cat. Effect of vasopressin on renal arterial blood flow (measured in one of left renal arteries with an electromagnetic flowmeter). 240, 130, 60 and 130 m-u. into renal artery at arrows. *B*, 2·3 kg cat. Effect of repeated doses of vasopressin on mesenteric venous blood flow (electromagnetic flowmeter). At arrows 50, 200, 500 and 100 m-u. I.V.

Femoral blood flow

Angiotensin. Injections of angiotensin into the femoral artery caused large decreases in femoral arterial blood flow (0·01–1 μg in two cats, Fig. 7*B*). The results of intravenous injections on femoral venous flow in 8 experiments were variable (Fig. 7*A*). Sometimes there were two different types of response in the same experiment, consisting either of a decrease in blood flow, a rise in blood flow, or a fall, a rise and then a second fall. The increases in flow were usually less than those caused by comparable doses of adrenaline in the same cat and the time relation of the increase was also usually different. When there was a delayed rise in femoral venous blood flow it coincided with the fall and secondary rise in the blood pressure (Fig. 7*A*). In one cat an intravenous infusion (3 μg in 1 min 40 sec) of angiotensin caused a small decrease in femoral venous blood flow, and in another cat a similar infusion (0·9 μg in 2 min) caused a slight increase in flow.

Vasopressin. In four cats intravenous injections of vasopressin (30–240 m-u.) caused a slowly developing prolonged decrease in femoral venous blood flow. Injections into the femoral artery caused a decrease in femoral arterial blood flow (6–90 m-u. in one cat).

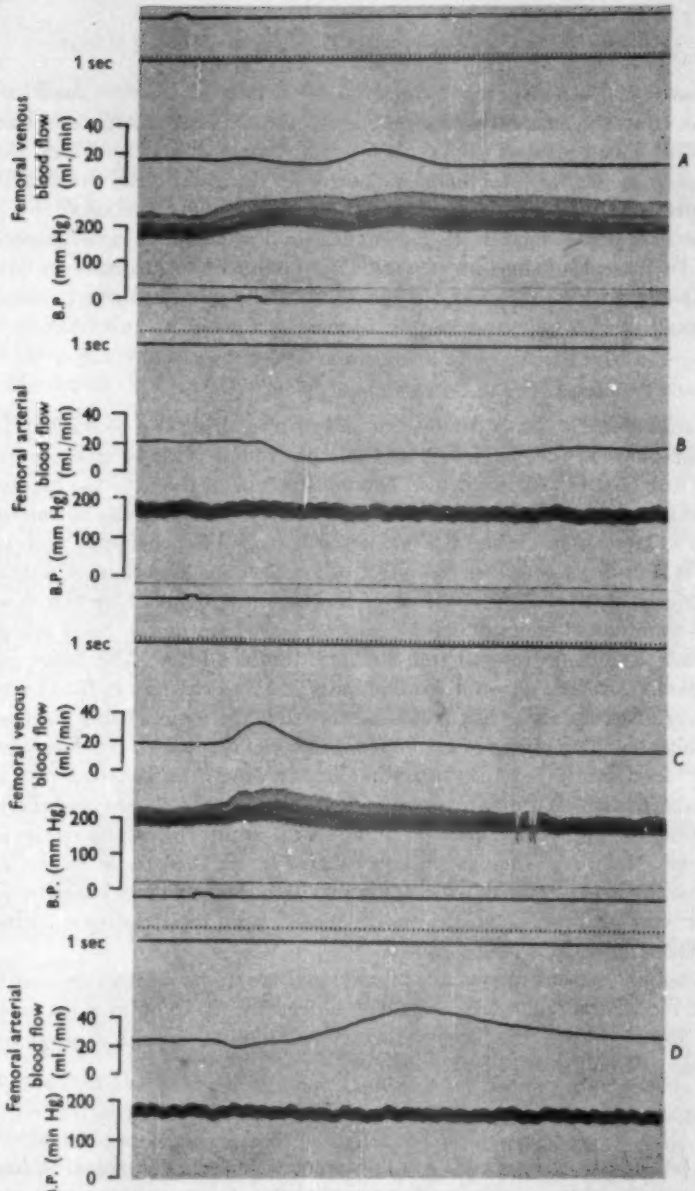

Fig. 7. Femoral blood flow (electromagnetic flowmeter). *A*, 4·2 kg cat; femoral *venous* blood flow, angiotensin 2 μg I.V. at signal. *B*, 3·5 kg cat, femoral *arterial* blood flow, angiotensin 0·05 μg into femoral artery at signal; *C*, 4·2 kg cat (same cat as *A*); femoral *venous* blood flow adrenaline 3 μg I.V. at signal; *D*, 3·5 kg cat (same cat as *B*); femoral *arterial* blood flow adrenaline 0·2 μg into femoral artery at signal.

Adrenaline. Both intravenous injections (0·025–10 μg in seven cats) and injections into the femoral artery (0·2 μg in one cat) caused large increases in femoral blood flow (Fig. 7*C*, *D*). After intravenous injections the increase in blood flow was usually shorter in duration than the rise in blood pressure and it was often followed, occasionally preceded, by a decrease in femoral blood flow. The increase in flow was always the greater effect. In two cats infusions of adrenaline gave rise to increases in flow which lasted only a short time compared with the sustained rise in blood pressure (2 μg in 2 min, and 9 μg in 1¼ min).

Carotid blood flow

No reliable method has yet been found of measuring the blood flow to the brain alone in the cat, but it was thought worth-while to measure the carotid flow in a few experiments. The venous flow in the external jugular veins was measured in two cats but in others the insertion of the flowmeter seemed to arrest flow. When the flowmeter is placed in continuity with an artery it is difficult to assess the effect of intravenous injections of a drug on that region, because its arrival in the region is delayed by the flowmeter circuit until changes have occurred elsewhere in the body which may alter the blood pressure and therefore the blood flow. The delay can be estimated, so that account is taken only of those changes in blood flow occurring after the drug has had time to reach the region being studied.

Angiotensin. Injections of angiotensin into the carotid artery decreased carotid blood flow (0·1–1 μg in two cats). Intravenous injections (0·2–2 μg in eight cats) caused an initial increase in flow attributed to the general rise in blood pressure and then a decrease in flow or a return to the original level. Sometimes the initial increase in flow seemed larger than could be readily explained by delay in the flowmeter circuit. In one experiment the external jugular venous flow increased, or increased and then decreased after intravenous injections (2–2·6 μg).

Adrenaline. After intravenous injections there was a preliminary rise in carotid blood flow, followed usually by a decrease (3–10 μg in eight cats). When injected into the carotid artery adrenaline caused a profound decrease in blood flow (2 μg in one cat).

Coronary blood flow

The left coronary arterial blood flow was measured in five cats. A long glass cannula whose central end was connected to a carotid artery was passed down the left subclavian artery through the aorta into the orifice of the main left coronary artery (Dawes, 1947). When possible the cannula was tied in position by a ligature previously placed round the left coronary

artery. The latter sometimes branched almost at once and it was easy to obstruct one of the branches with the cannula tip.

In all five cats injections of angiotensin (1–3 μg) into the loop feeding the coronary artery caused a slight decrease in coronary blood flow followed by a rise in blood pressure.

Pulmonary arterial pressure and pulmonary vascular resistance

Angiotensin. In six cats in which the flow and pressure were measured in the main pulmonary artery small doses of angiotensin (0·2–2 μg I.V.), sufficient to raise the systemic blood pressure, often caused no change in pulmonary arterial pressure. With larger doses of angiotensin pulmonary arterial pressure usually rose although often more briefly than systemic blood pressure. The rise in pulmonary arterial pressure sometimes coincided with the second peak in the systemic blood pressure. In another seven cats blood flow and pressure were measured in the left pulmonary artery and the left atrial pressure was also measured (Barer & Nüsser, 1957). Injections of angiotensin (0·6–5 μg) into the left pulmonary artery caused an increase in pulmonary vascular resistance (44–119 %) in five cats, and very small decreases accompanied by an increase in flow in the other two cats. The most common result was a decrease in left pulmonary blood flow, no change or a slight rise in pulmonary arterial pressure and no change or a slight rise in left atrial pressure.

Vasopressin. In three cats in which the flow and pressure were measured in the main pulmonary artery doses of vasopressin (60–300 m-u.) which raised the systemic blood pressure had no significant effect on the pulmonary arterial pressure. In four other cats there was no significant change in pulmonary arterial pressure, blood flow to the left lung and left atrial pressure after injections of vasopressin (60–300 m-u.) into the left pulmonary artery, but in three of these the systemic pressure was also unaffected.

Adrenaline. The pulmonary arterial pressure was always raised by intravenous doses of adrenaline which caused a rise in blood pressure. The increases were greater than after comparable doses of angiotensin. In five cats the flow and pressure in the left pulmonary artery and (in three cats) the left atrial pressure was measured. Injections of adrenaline (2–20 μg) into the left pulmonary artery caused a decrease in calculated pulmonary vascular resistance, the blood flow increased, the pulmonary arterial pressure rose slightly or fell slightly and the left atrial pressure rose slightly.

DISCUSSION

The three pressor substances, angiotensin, vasopressin and adrenaline, differed greatly in their actions on the circulation of the cat. The results of adrenaline injections, which were obtained for the purpose of comparison, are similar to those obtained by other methods and in other species. There was a large increase in cardiac output and a redistribution of blood to muscular regions.

Angiotensin caused no regular change in cardiac output in the cat, so that the rise in systemic blood pressure is attributed to increased peripheral resistance. Angiotensin increased resistance in every vascular bed into which it was injected directly, but in some regions there was sometimes an increase in flow after intravenous injections. If the blood flow to the kidney and gut is always reduced by angiotensin and the cardiac output remains constant, it follows that other regions such as that supplied by the femoral artery will receive more blood in spite of partial vasoconstriction. Thus there is some redistribution of blood flow. The variable changes in femoral blood flow after intravenous injections of angiotensin can thus be explained in terms of weak local vasoconstriction combined with a rise in blood pressure, the time relations of the two processes being different. The possibility also cannot be excluded that small portions of the vascular bed supplied by the femoral and carotid arteries may be dilated by angiotensin. Angiotensin had a mild constrictor action on the coronary and pulmonary blood vessels, and probably some action on the hepatic vessels. Some of the delayed changes in cardiac output and regional blood flow after angiotensin may also have been caused by reflexes initiated by the rise in blood pressure.

The results are in agreement with those of earlier workers, who concluded that angiotensin extracted from natural sources caused vasoconstriction in all systemic vascular beds (Braun-Menéndez, 1956; Green & Kepchar, 1959). Similarly, synthetic angiotensins caused vasoconstriction in the rabbit's ear and hind paw (Meier, Tripod & Studer, 1958), the coronary and femoral arteries of dogs (Bianchi, de Schaepdryver, de Vleeschhouwer & Preziosi, 1960), and the skin and muscle of man (Bock & Krecke, 1958; Bock, Krecke & Kuhn, 1958; Bock, Dengler, Krecke & Reichel, 1958). The latter also observed an antidiuretic effect and changes in K^+ excretion with extraordinarily low concentrations of angiotensin. However, angiotensin had no local effect comparable with that of adrenaline on the wall of the carotid sinus (McCubbin, Page & Bumpus, 1957; Bianchi *et al.* 1960).

There have been a number of reports suggesting that, in man, angiotensin causes a *decrease* in cardiac output. Using natural angiotensin, Bradley & Parker (1941) and Wilkins & Duncan (1941) concluded from

ballistocardiograms that there was a decrease in minute volume. Using synthetic isoleucine angiotensin, Gersmeyer, Spitzbarth & Weyland (1958) calculated from measurements of the pressure pulses that the minute volume decreased, mainly owing to bradycardia. G. de J. Lee (personal communication) showed that synthetic valine angiotensin caused a fall in cardiac output of 24% in normal males, using the dye method; in three other subjects, using the Fick method, a fall of 12% was found. In both series the fall in cardiac output was mainly due to bradycardia; the systemic vascular resistance increased by 70%. However, Sancetta (1960) using purified natural decapeptide angiotensin found no change in cardiac output measured by the Fick principle and no change in pulmonary vascular resistance. Thus there seems to be some difference between the effects of angiotensin on the cardiac output of anaesthetized cats and most of the reported effects on unanaesthetized man. There is also a discrepancy between the observations on man by Sancetta (1960) and on dogs by Bianchi *et al.* (1960), who found the pulmonary blood vessels insensitive to angiotensin, and those on man by Lee (personal communication), who calculated that some pulmonary vasoconstriction had taken place. The more direct observations on cats support the latter conclusion, although the pulmonary blood vessels appeared to be less sensitive to angiotensin than the systemic vessels.

Vasopressin had no constant effect on the cardiac output in cats; all the vascular beds examined were constricted by it except the renal, which was greatly dilated. Previous work also has shown that vasopressin is a powerful vasoconstrictor (Pickford, 1952; Thorn, 1958), except for one observation that the pial vessels were dilated (Forbes, Finley & Mason, 1933) and some conflicting observations on the kidney. Walker, Schmidt, Kendall & Johnson (1937) observed a large rise in renal blood flow (measured with a Rein type of thermostromuhr) after injection of Pitressin (vasopressin; Parke, Davis) in 3 out of 20 unanaesthetized dogs and rabbits. Handowsky & Samaan (1937), also using a thermostromuhr, observed an initial reduction followed by a large increase in renal blood flow after large but not after small doses of Pitressin in unanaesthetized dogs. Wakim, Herrick, Baldes & Mann (1942) found that Pitressin caused a profound decrease in renal blood flow measured with a similar instrument in conscious and anaesthetized dogs, but in the former there sometimes followed a prolonged rise. In 1946 Dicker & Heller showed that either Pitocin (oxytocin; Parke, Davis) 3 m-u./kg, or Pitressin 0·3 m-u., but not Pitressin 3 m-u./kg caused large increases in renal plasma flow and glomerular filtration rate derived from inulin and diodone clearances. They explained their results on the grounds of contamination of the one hormone by the other. Maxwell & Breed (1951) found that large but not

small doses of Pitressin reduced the renal plasma flow and glomerular filtration rate measured by clearance methods in man. Brooks & Pickford (1958) demonstrated by renal clearance methods that highly purified oxytocin and synthetic oxytocin caused powerful renal vasodilatation in conscious dogs which was prevented by the injection of arginine vasopressin; arginine vasopressin alone did not affect renal plasma flow. Ali (1958), also by renal clearance methods, showed that lysine vasopressin did not prevent the increase in renal plasma flow caused by oxytocin, but he did not record the effect of lysine vasopressin alone. Demunbrun, Keller, Levkoff & Purser (1954) showed, again by clearance methods, that highly purified oxytocin could restore to normal the renal plasma flow, glomerular filtration rate and tubular maxima which had been profoundly reduced by hypophysectomy. Most of these results can be explained on the grounds that oxytocin is a powerful renal vasodilator which is antagonized in this as in other respects (Brooks & Pickford, 1958) by vasopressin, that vasopressin sometimes causes renal vasoconstriction, and that natural extracts may contain mixtures of the two hormones. The present results on lysine vasopressin (the natural hormone in the hog) suggest that it differs functionally as well as chemically from the hormone of other species. If, as is thought probable, vasopressin and oxytocin are normally released together, this transfer of functions from the one hormone to the other in the hog compared with other species would be less significant. There seems to be general agreement that the changes in renal blood flow caused by posterior pituitary hormones cannot be correlated with their effects on urine formation.

SUMMARY

1. In cats anaesthetized with chloralose the actions of synthetic angiotensin, synthetic lysine vasopressin and adrenaline on blood pressure, right heart output and regional blood flow have been compared.

2. There were no constant changes in cardiac output after angiotensin and vasopressin but adrenaline caused a rise.

3. The rise in blood pressure caused by angiotensin and vasopressin was mainly due to increased peripheral resistance. Adrenaline usually caused a biphasic increase in total peripheral resistance so that at different times during the rise in blood pressure this was predominantly due to increased cardiac output or to increased peripheral resistance.

4. Angiotensin constricted the renal and mesenteric vascular beds strongly, the femoral carotid pulmonary and coronary vascular beds probably less strongly. The existence of small vascular beds which could be dilated by angiotensin could not be excluded. The pulmonary vascular

bed appeared to be less sensitive to angiotensin than the systemic vascular bed.

5. Lysine vasopressin constricted the mesenteric and femoral vascular beds, had no significant effect on the pulmonary vascular bed, and caused a profound dilatation in the renal vascular bed.

6. Adrenaline constricted the renal and carotid vascular beds; there were variable effects on the mesenteric vessels. The femoral area was both dilated and constricted, the former predominating. The pulmonary arterial pressure was raised much more than by comparable doses of angiotensin, but the pulmonary vascular resistance was reduced owing to a combination of factors.

7. As far as a comparison is possible, angiotensin appears to be five to ten times as powerful, weight for weight, in raising the blood pressure as adrenaline in the cat.

The generous gifts of angiotensin (synthesized by Ciba, Basel) from Professor Sir George Pickering and of lysine vasopressin from Professor Vincent du Vigneaud are acknowledged with much gratitude. I am very grateful to Dr G. S. Dawes for continuous help and criticism of both the work and manuscript and to Professor Sir George Pickering for his helpful advice. Dr D. G. Wyatt has provided invaluable help on physical problems and been most generous in the loan of his instrument. Mr A. Ryder, Mr H. Eldridge and Mr G. Smith are thanked for their expert technical assistance.

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EFFECTS OF HYALURONIC ACID UPON DIFFUSION OF SOLUTES AND FLOW OF SOLVENT

BY A. G. OGSTON* AND T. F. SHERMAN†
From the Department of Biochemistry, University of Oxford

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Day (1950, 1952) showed that hyaluronic acid plays an important part in the resistance of connective tissue to the flow of fluid through it. Other workers (Krogh, 1919; Wright, 1934; Harris & Burn, 1949; Paulson, Sylven, Hirsch & Snellman, 1951) have found that the diffusion of solutes occurs considerably more slowly through connective tissue than through water: this may be due in part to hyaluronic acid, which is known (Meyer, Davidson, Linker & Hoffman, 1956; Rogers, 1961) to be widely distributed in connective tissues. Ogston & Phelps (1961) have shown that hyaluronic acid has an important effect on the distribution of solutes between buffer solutions and those containing hyaluronic acid, and that the mechanism of this effect is probably steric.

The heterogeneity of samples of connective tissue would make it difficult to relate effects on diffusion or flow to their composition. We therefore decided to make measurements on homogeneous systems, particularly on solutions of hyaluronic acid, whose compositions are exactly known: concentrations of hyaluronic acid were used which were not greater than those known to exist in one type of connective tissue, Wharton's jelly (Sherman, personal communication). Serum albumin, synthetic polyglucose, and glucose were used as representative diffusing solutes. The results, though preliminary and needing extension, show that hyaluronic acid has important effects on both diffusion and flow and therefore presumably plays a part in determining the permeability of connective tissue.

METHODS

Measurement of diffusion coefficients. Diffusion runs were carried out with a cylindrical Perspex cell (Fig. 1) containing two compartments (upper and lower) of 1 cm internal radius. The lower compartment (height 0·5 cm) was separated from the upper compartment by a Millipore (Millipore Filter Corporation) HA-type membrane (pore size 0·45 mμ), which was sealed in place with 'Evostik' (Erode Ltd), rubber adhesive; the lower compartment could

* Present address: Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T.
† Present address: Department of Zoology, Oberlin College, Oberlin, Ohio, U.S.A.

be filled by means of a syringe inserted through an opening (fitted with a screw plug) on the side of the chamber. During a diffusion run the solution in the bottom chamber remained unstirred, but the top chamber was stirred by a glass rod rotated by a small electric motor, which was mounted in such a way that no heat or vibration was transferred from the motor

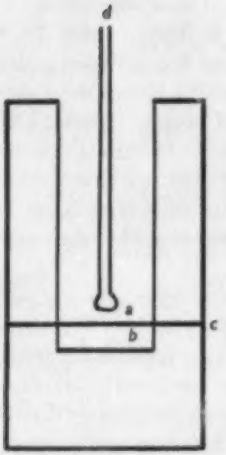

Fig. 1

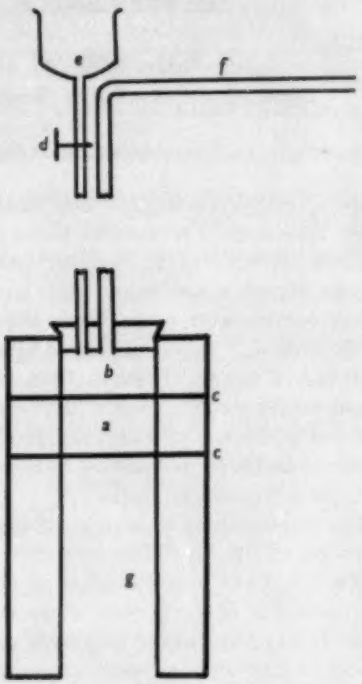

Fig. 2

Fig. 1. Diagram of diffusion cell. *a*, Top chamber, which contains buffer solution; *b*, lower chamber, which contains diffusing solute and buffer (or hyaluronic acid) solution; *c*, Millipore HA membrane; *d*, stirrer.

Fig. 2. Apparatus for measuring flow of solvent through solutions of hyaluronic acid: The hyaluronic acid solution is placed in chamber *a* between the Millipore membranes *c*; chamber *a* is filled by means of a syringe through a screw plug (not shown); chamber *b* is filled with buffer solution through funnel *e* until a meniscus reaches the horizontal part of tube *f*; the rubber tube from *e* is then closed with clip *d*. The buffer solution flows from *f*, through chambers *b* and *a* and membranes c, trickling out through *g*, under a total head of 25·7 cm of water. The rate of flow is obtained from the movement of the meniscus in *f*.

to the diffusion cell. The apparatus was used at room temperature, but the temperature of the room in which it was used varied only slightly, and the apparatus was located in a protected corner away from any sources of heat or possible draughts. The thick (1 cm) Perspex wall of the cell provided considerable insulation from temperature changes.

A thermometer was placed just to one side of the outer wall of the cell, and during the course of a 1 hr run the air temperature did not change by more than 0·3° C.

The procedure for a diffusion run was as follows: The cell was held horizontally and the bottom chamber was filled by a syringe with the buffered solution ((M) Na_2HPO_4 0·0077, KH_2PO_4 0·0023, NaCl 0·2; pH 7·3). This solution contained either (*a*) the diffusing substance alone, or (*b*) the diffusing substance plus hyaluronic acid complex. The cell was then rotated to its normal vertical position, and the top of the membrane separating the two chambers was swabbed with cellulose tissue. The cell was placed under the stirrer, the end of which was fixed about 3 mm above the membrane. After a 5 min period the stirrer was started, and, at zero time, 4 ml. of buffer solution was pipetted into the top chamber, care being taken to run the solution down the side of the chamber wall to prevent undue disturbance of the membrane. At the end of a given period of time, the cell was quickly removed and tilted to pour off the contents of the top chamber; this solution was then analysed for the diffusing substance. It was found that the most reproducible results were obtained when the main diffusion run was preceded by two very short runs (usually 3 min each), the membrane being swabbed dry after each, and the result obtained from the second of these short runs being subtracted from that of the main run.

The experimentally determined data were, therefore, the concentrations of diffusing substance found in the solution in the top chamber after a measured length of time. This concentration was expressed in the same terms as those used for the original solution in the lower chamber; for dinitrophenol (DNP)-labelled serum albumin this was expressed as absorption (at 360 mμ), for ^{14}C-labelled polyglucose as counts $min^{-1}.ml.^{-1}$, and for glucose as mg/ml. The approximate initial concentrations used were: DNP-labelled serum albumin, 1·25–2 g/100 ml.: polyglucose, less than 1 g/100 ml.

Interpretation of the diffusion measurements. The method used is based upon the diffusion of material out of a semi-infinite medium into one (the stirred compartment) in which the concentration is maintained uniform. The actual case is, however, more complex than this in three respects:

(i) The medium from which diffusion occurs is not infinite in extent. The effects of this were minimized by choosing diffusion times such that the concentration of solute at the distant boundary would have changed at the end of the experiment by less than 5% from the initial value.

(ii) The concentration in the stirred compartment increases with time. Allowance is made for this by successive approximation by means of equations 3·13 to 3·21 of Crank (1956). This leads to the expression

$$M = 2AC\,[2\,(Dt/\pi)^{\frac{1}{2}} - ADt/V + 4A^2CD^{\frac{1}{2}}\,t^{\frac{3}{2}}/3V^2\pi^{\frac{1}{2}} - \ldots], \qquad (1)$$

where M is the amount of material that has diffused out of the unstirred compartment at time t, C is the initial concentration in the unstirred compartment, A the cross-sectional area, V the volume of the stirred compartment, and D the diffusion coefficient in the unstirred compartment. Under the conditions used, the third and higher terms of equation (1) were insignificant, and equation (1) could, therefore, be solved directly as a quadratic in $D^{\frac{1}{2}}$.

(iii) The medium from which diffusion is taking place is compound, consisting of a membrane and hyaluronic acid solution. The value of D obtained by equation (1) is, therefore, an apparent value D_x which is compounded of the value in the membrane (D_1) and that in the hyaluronic acid solution (D_2). According to equation 3·57 of Crank (1956),

$$2\left(\frac{D_x t}{\pi l^2}\right)^{\frac{1}{2}} = 2\left(\frac{D_1 t}{\pi l^2}\right)^{\frac{1}{2}}\left[1 + 2\sum_{n=1}^{\infty} \alpha^n e^{-(n^2 l^2/D_1 t)}\right] - 4\sum_{n=1}^{\infty} n\alpha^n \operatorname{erfc}\frac{nl}{(D_1 t)^{\frac{1}{2}}}, \qquad (2)$$

where l is the thickness of the membrane, $\alpha = (1-[D_1/D_2]^{\frac{1}{2}})/(1+[D_1/D_2]^{\frac{1}{2}})$ and erfc = 1 − erf. (erfz = the error function $= \frac{2}{\sqrt{\pi}}\int_0^z e^{-\eta^2} d\eta$, see Crank loc. cit.) Under the conditions of time used, the approximation given by Crank (equation 3·58) could not be used.

In practice therefore, two measurements were always made with the same membrane: the first with the solute in buffer solution, the second with the solute in hyaluronic acid solution. In the first experiment, when the diffusion coefficient in buffer solution (D_2) is known the value of that for the membrane (D_1) is computed from the measured value of D_x by means of equation (2); this involves comparison of the left-hand side of the equation with the right-hand side, choosing a range of assumed values of D_1 and interpolating the value of D_1 which makes the two sides equal: the sum terms in equation (2) converge rapidly and values of n only up to 3 are needed. In the second experiment, the same value of D_1 is assumed to hold; the right-hand side of equation (2) is then computed for a range of values of D_2 (i.e. of α) and the value of D_2 is obtained by interpolating the measured value of the left-hand side in the plot of the value of the right-hand side against α. (It should be noted that Crank's Fig. 3·8 could not be used for this purpose; Dr Crank agrees with us that an error was made in computing the curves in that figure, other than that for $D_1/D_2 = 1$). This method of calculating D_2 is not strictly valid (though the results are likely to be approximately correct), since equation (1) applies strictly to diffusion out of a single homogeneous medium.

The diffusion coefficients calculated by means of this equation were corrected to 20° C (from the temperature, T, at which the run was carried out) by using the following expression:

$$D_{20} = D\left(\frac{293{\cdot}2}{T}\right)\left(\frac{\eta}{\eta_{20}}\right),$$

where η/η_{20} is the ratio of the viscosity of water at the experimental temperature to the viscosity of water at 20° C. The temperature correction factor is about 2·5% per degree deviation from 20° C.

Measurement of flow through solutions of hyaluronic acid complex. The apparatus (Fig. 2) consisted essentially of three Perspex rings (1 cm internal radius), the middle one of which (height 1·2 cm) was separated from the others by Millipore HA-type membranes sealed with 'Evostik' cement. The top cylinder was connected to a filling tube and to an L-shaped piece of glass tubing, the latter of which served to increase the height of the column of fluid (from the bottom of the middle chamber) to 25·7 cm, and to maintain steady this pressure head during the course of an experiment. Flow was measured by timing the excursion of a meniscus along the horizontal arm of this tube; the tube was calibrated by weighing the mercury which occupied a measured length of the tube. Experiments were performed by filling the middle chamber with a solution of hyaluronic acid complex (or of buffer solution alone) by means of a syringe inserted through a side hole, by filling the top chamber with buffer solution (see Methods) through the filling tube, and by measuring the flow rate in the horizontal arm.

Isolation of hyaluronic acid complex. This was accomplished by the ultrafiltration method described by Ogston & Stanier (1950). Synovial fluid was collected from the astragalotibial joints of oxen soon after death, and was centrifuged in a Spinco model-L ultracentrifuge at 73,000 *g* for 60 min. to remove particulate matter. Hyaluronic acid–protein complex (constituting approximately 3 parts by weight of hyaluronic acid to 1 part of protein) was separated by filtration, with about nine washings, at 4° C, on a 5-on-3 sintered-glass filter of 1 μ (or less) average pore diameter (Baird and Tatlock Ltd). The buffer solution used for washing was composed as described in Methods. The complex was finally dissolved in this buffer solution to a much lesser volume than the original synovial fluid, and was centrifuged at 73,000 *g* for about 60 min.

Preparation and estimation of dinitrophenol (DNP)-labelled serum albumin. Preparation was carried out by the method described by Ogston & Phelps (1961). DNP-labelled serum albumin was estimated by measuring the extinction of the solution at 360 mμ. For the diffusion measurements, concentrations of the DNP-labelled serum albumin were expressed in terms of the absorption at 360 mμ, although this could be approximately translated to g/ml. from the knowledge of the extinction of the original preparations; thus a preparation

of approximately 0·93 g/100 ml. (calculated from the volumes used in preparation) DNP-labelled serum albumin had an extinction of 5·1 in a 1 cm light-path.

^{14}C-labelled polyglucose and its estimation. ^{14}C-labelled polyglucose was kindly supplied by Mr C. F. Phelps from material prepared by a cyanohydrin reaction of acidified ^{14}C-labelled KCN with polyglucose. The ^{14}C-labelled polyglucose was exhaustively dialysed before use to remove any small molecular components. The material, though polydisperse, had an estimated weight average molecular weight of about 33,000.

Estimation of ^{14}C-labelled polyglucose consisted solely of measuring the radioactivity of the unknown solution, as concentration of polyglucose was expressed in the diffusion measurements in terms of radioactivity. The radioactivities of dried samples of the unknown solutions were measured with a Geiger-Muller counter of end-window type; the original solution had a measurable radioactivity of 184,000 counts/min.ml.

Estimation of glucose. This was done by the method of Nelson (1944) and Somogyi (1945), exactly as described by them.

RESULTS

Diffusion coefficients

Preliminary tests. The lower solution consisted of a 2 % gelatin gel, containing glucose or sodium chloride; no membrane was used. The apparent diffusion coefficients agreed closely with published values in water; it is to be expected from the data of Friedman & Kraemer (1930) that 2 % gelatin would have little effect on the diffusion of small molecules.

Diffusion coefficients in hyaluronic acid. The results are given in Table 1. D_2 values could not be calculated for polyglucose, as no value of its diffusion coefficient in water was available. The diffusion coefficient (at 20° C) of serum albumin was taken as $6{\cdot}1 \times 10^{-7}$ cm^2/sec (Kekwick, 1938), and that of glucose as $5{\cdot}9 \times 10^{-6}$ cm^2/sec (Friedman & Carpenter, 1939). A single experiment was also done on the diffusion of serum albumin in water and in a gel made by heating a 0·3 % solution of soluble collagen to 37° C; the values of D_x were almost identical, showing that collagen gel of this concentration has little effect.

Flow through solutions of hyaluronic acid. The results are given in Table 2.

DISCUSSION

Diffusion of solutes through hyaluronic acid. Because of the configuration of hyaluronic acid, as a flexible chain polymer of very great molecular weight (Blumberg & Ogston, 1958), even a very dilute solution of it consists of a continuous entangled meshwork rather than of separate molecules (see Balazs, 1958). Ogston & Phelps (1961) have shown that the effect of hyaluronic acid on the equilibrium distribution of solutes is consistent with this picture. It should be expected that randomly-moving diffusing particles would meet with resistance from such a structure, the greater the higher the concentration of hyaluronic acid (that is, the smaller the mesh) and the larger the diffusing particles. Our measurements support these

TABLE 1. Diffusion coefficients of solutes in solutions of hyaluronic acid

Expt. no.	C_{HAC}	Solute	C_v	t (min)	M/V	T	$10^7.D_x$ (cm²/sec)	$10^7.D_2$ (cm²/sec)	D_2/D_w
I	0	Serum albumin	6·37	55	0·181	21·2	3·17	6·1	1·00
	0·2		6·40		0·155	21·3	2·28	1·63	0·27
	0·4		6·40		0·144	21·2	1·97	0·94	0·15
	0·6		6·52		0·133	21·2	1·61	0·45	0·07
	0·8		6·40		0·123	21·0	1·44	0·28	0·05
II	0	Serum albumin	10·75	57	0·240	20·0	1·92	6·1	1·00
	0·3		10·75		0·219	20·3	1·58	2·09	0·34
	0·6		10·75		0·205	20·5	1·38	0·95	0·16
III	0	Polyglucose	19,380	180	1730*	21·3	10·7	—	—
	0·25		19,380		1475*	21·2	7·6	—	—
IV	0	Glucose	10·0	27	0·396	20·4	13·0	59·0	1·00
	0·2		10·0		0·390	21·5	12·2	43·7	0·74
	0·4		10·0		0·391	21·6	12·3	44·6	0·76
	0·6		10·0		0·376	22·0	11·2	30·2	0·51
	0·8		10·0		0·358	22·1	10·2	20·9	0·35
V	0	Serum albumin	10·03	57	0·215	19·0	1·81	—	—
	0·3†		10·01		0·211	20·0	1·71	—	—

C_{HAC}, concentration of hyluronic acid complex.

t, duration of diffusion run.

M/V, final concentration of solute in top compartment expressed as follows: DNP—serum albumin, extinction at 360 mμ; ^{14}C—polyglucose, counts/min/ml.; glucose, mg/ml.

C_v, initial concentration of solute in same units as M/V.

T, temperature of diffusion run.

D_x, apparent diffusion coefficient at 20° C.

D_2 diffusion coefficient corrected for membrane.

D_2/D_w, diffusion coefficient in hyaluronic acid solution/same in buffer solution.

* These values represent the mean of five runs; standard deviation for both groups was 3 %. † This experiment was done with a collagen gel, with no hyaluronic acid present; concentration given is that of collagen.

TABLE 2. Rate of buffer flow through solutions of hyaluronic acid

Concentration of hyaluronic acid (g/100 ml.)	Flow rate
0	1 cm/sec = 0·03 ml./sec
0·28	1 cm/235 sec = $12·1 \times 10^{-5}$ ml./sec
0·56	1 cm/324 sec = $8·8 \times 10^{-5}$ ml./sec
0·84	1 cm/445 sec = $6·4 \times 10^{-5}$ ml./sec

qualitative predictions. It is tempting to try to provide a more quantitative interpretation but this would not be justified at present; the results which we have are too few and, as their variation between experiments shows, not accurate enough. This inaccuracy is due in part to the need for estimating D_2 from D_x, with the assumption that D_1 does not change between successive experiments; as Table 1 shows, a variation of D_x implies a larger variation of D_2, whose value is, therefore, rather sensitive

to that of D_1. It is hoped to extend and improve the accuracy of such measurements, perhaps by using a steady-flow method.

Flow through hyaluronic acid solution. The experiments described compare the rate of flow of solvent (water plus buffer salts) through two Millipore membranes, with the flow of solvent when the region between the membranes is occupied by solutions of hyaluronic acid. In the former case, the solvent between the membranes flows through a wide cylindrical tube, without other hindrance than that of its walls, which is negligible. In the latter case, flow of solvent occurs through the hyaluronic acid meshwork, which is itself immobilized by the lower membrane: the very greatly reduced rates of flow show that the immobilized hyaluronic acid is here playing a major part in providing resistance to the flow of solvent. Sullivan & Hertel (1942) have treated the similar case of flow of fluid through porous plugs by considering the plug as equivalent to a set of narrow tortuous capillaries; but again the application of a detailed theory does not seem worth while at present.

Physiological considerations. Both sets of experiments show qualitatively that hyaluronic acid (or material of similar configuration) can be expected to have large effects both on the diffusion of solutes in tissue spaces and on the flow of fluid through them. A large part of the resistance to exchange between blood capillaries and cells or lymphatic capillaries may thus reside in the spaces themselves rather than in the vessel walls. The large effect of hyaluronidase on the flow of fluid in connective tissue, and Day's (1952) experiments on flow through fascia, are understandable in these terms. Quantitative assessment of this contribution to resistance will need further investigation.

It is interesting that a collagen network alone has relatively little effect on diffusion. Since its fibres are much thicker than those of hyaluronic acid (500–1000Å as compared with about 8 Å) it forms, at comparable concentration, a much coarser meshwork through which both diffusion and flow can occur relatively easily. A collagen network can, however, as Fessler (1957) has pointed out, serve to immobilize hyaluronic acid by penetrating the hyaluronic acid meshwork and so, at second hand, to immobilize water and solutes.

SUMMARY

1. The rates of diffusion of glucose, polyglucose and serum albumin in solutions of hyaluronic acid have been measured; the hyaluronic acid markedly reduces the diffusion rates, increasingly with the concentration of hyaluronic acid and the molecular sizes of the diffusing solutes. A gel of collagen has little effect on diffusion.

2. Hyaluronic acid is shown to have a large effect on the bulk flow of solvent through solutions which contain it.

3. The physiological implications of these results are briefly discussed.

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ACCOMMODATION IN MOTONEURONES OF THE RAT AND THE CAT

BY K. BRADLEY AND G. G. SOMJEN

From the Department of Physiology, University of Otago, Dunedin, New Zealand

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The response of motoneurones to slowly changing or steady stimuli is of interest for the understanding of their function, because ventral horn cells are thought to be continually subject to a varying degree of excitation and inhibition. Fuortes & Hubel (1956), Fuortes (1957) and Kolmodin & Skoglund (1958) considered that sustained facilitation as well as rhythmical reflex firing are due to a steady synaptic drive. Frank & Fuortes (1956) have observed slow spontaneous changes in the level of the membrane potential of a motoneurone more or less paralleled by variations of its electric threshold, and in the same paper described experiments indicating that motoneurones of the cat accommodate to subliminal d.c. pulses only to a very limited degree. This is in keeping with the implications of earlier experiments by Barron & Matthews (1938) and by Fuortes (1954) showing that 'ascending polarization' with direct currents can cause continued firing of ventral horn cells. However, Araki & Otani (1959) found appreciable accommodation of single motoneurones in the toad to exponentially blunted current pulses. These authors found two types of response which they attributed to genesis of the impulse at different loci within the cell, the initial axonal segment when accommodation was fast, and the soma-dendritic membrane when it was slow.

The main subject of the study presented in this paper was the response of single mammalian motoneurones to linearly rising ('triangular') and suprathreshold constant-intensity ('rectangular') current pulses. In addition, the accommodation of single motor axons has been studied by intracellular stimulation, and also by stimulating ventral roots or peripheral nerves and using the antidromically conducted spike recorded in the impaled central portion of the motoneurone to monitor the unitary response.

A preliminary report of some of the results has already appeared (Somjen & Bradley, 1959).

METHODS

White or hooded rats weighing 300–400 g were anaesthetized with pentobarbitone (Sagatal, May and Baker; 65 mg/kg body weight intraperitoneally, initially, supplemented if necessary with small additional doses at about 2–4 hr intervals). Laminectomy was performed over the entire lumbar section of the vertebral column, and the spinal roots from L3 to L6 were cut at their exit from the vertebral canal and dissected up to near their junctions with the cord. L4 and L5 segments were selected in most experiments for the purpose of recording and stimulation, because of the suitable size and length of the roots, which in rats are the main origin of the branches of the sciatic nerve to the leg. In some experiments the ventral roots were left intact and the sciatic nerve in the thigh was prepared for antidromic stimulation. The pia mater was peeled off the dorsolateral surface of the cord at the site chosen for micro-electrode entry. To limit venous bleeding during the laminectomy the rats were held entirely suspended, with clamps holding the head, the spinous process of the vertebra prominens, and the root of the tail. Two further pairs of clamps were inserted after completion of the laminectomy, one pair holding the body of the vertebra on each side at the level of micro-electrode insertion, another two segments caudally. The limbs of the rat were now raised to the level of the animal's body so that the ventral skin was stretched, lending support to the abdominal contents. This manoeuvre, which is designed to prevent pooling of blood in dependent parts, was found essential for sufficient survival time of the preparation. To reduce movements of the spinal cord caused by venous pressure changes an open pneumothorax was then made on one side, D-tubocurarine (0·1 mg/kg body weight) injected intraperitoneally and artificial respiration started with 95 % O_2 + 5 % CO_2. A small Starling 'Ideal' pump was used, set to a rate of approximately 2 strokes/sec, and the volume was adjusted until excursions of the thorax were just visible. A paraffin pool was formed over all exposed nervous tissue and two small battery-powered heating coils encased in bent glass tubes kept the temperature of the paraffin between 37 and 38° C unless otherwise stated in the text. The dissection took about 2 hr.

Cats were used either in the acutely decapitate state or under pentobarbitone anaesthesia. The lower four lumbar vertebrae were opened and the appropriate dorsal and ventral roots severed and dissected up to the cord. L7 segment was usually used for micro-electrode recording. In some experiments the ventral roots were left intact and one or both branches of the popliteal nerve, or the nerve to the biceps-semitendinosus muscles, used for antidromic stimulation. Bilateral pneumothorax and full curarization ensured sufficient immobilization of the preparation for micro-electrode work. In the last experiments C-Toxiferin (Hoffman-laRoche) was used instead of tubocurarine, because this drug effects longer-lasting relaxation and its effect on the arterial blood pressure is negligible.

Single micropipettes filled with 3M-KCl and of 10–30 MΩ resistance were used both for recording from, and for passing current through the membrane of impaled motoneurones. A bridge circuit, similar to that described by Frank & Fuortes (1956) was used to minimize stimulus artifact. Potentials from impaled cells were fed via a cathode-follower input stage to conventional amplifiers, which were used AC-coupled and single ended in most experiments of the present series. The stimulator could provide linearly rising ('triangular') or d.c. ('rectangular') current pulses, or currents linearly rising to a plateau with an abrupt cut-off at the end. The rise time, the final strength and plateau duration of the current could be controlled independently; details of the stimulating circuit will be published elsewhere. A radio-frequency coupling unit kept the preparation isolated from the earth connexion of the stimulator. The stimulating current was monitored by taking off the potential between the junction point of the bridge circuit and ground (Frank & Fuortes, 1956, 'method 1') and displaying it after DC amplification on the second beam of the main recording unit. In those experiments where graded stimuli were applied to the ventral root a resistor of either 200 kΩ or 1 MΩ was placed in series with the root and the stimulus isola-

tion unit, and the potential across this resistance, or part of it, was displayed on the second beam for the purpose of measuring the stimulating current. All metal–fluid junctions carrying current were established by means of chlorided coarse silver wire.

RESULTS

General characteristics of records from rat motoneurones

It was found that the rat is a suitable animal for investigating the function of the spinal cord, particularly if the number of experiments wanted is relatively large and the required dissection simple. The preparations remain in good condition to provide at least 5 hr for recording, and single cells can be held impaled for periods of from 15 to 90 min. Motoneurones are about 30 μ in diameter in thionine-stained sections. On the average, the amplitude of the orthodromically or antidromically elicited spikes was smaller in rat than in cat motoneurones, but values up to about 100 mV have been observed, and it is likely that the smaller average amplitude is due to the fact that a larger proportion of cells is partially depolarized because rats' motoneurones are smaller than ventral horn cells of the cat. Resting potentials have not been systematically recorded, but Somjen & Gill (1958) found that although the mean resting membrane potential appeared smaller, values as high as the highest in cats' ventral horn cells were frequently encountered. The threshold for monosynaptic discharge in response to dorsal root stimulation appeared to vary between 4 and 15 mV. The duration of the positive after-potential (after-hyperpolarization) was shorter than in the cat, and lay between 30 and 75 msec (Fig. 1). Probably related to the relatively short positive after-potential is the tendency to fire repetitively at a rate faster than that usually found in cat motoneurones. Tetanic stimulation of the dorsal root at a frequency of 450/sec with shocks strong enough to elicit a large polysynaptic reflex caused some cells to fire up to 14 impulses in the first 100 msec. The rate of firing tended to slow down, but fairly high average frequencies (e.g. 50/sec) could be maintained in response to dorsal root tetanization lasting for 1 sec. In other motoneurones, however, it was difficult or impossible to evoke sustained repetitive discharge by dorsal root tetanization. Strength–latency curves for responses to rectangular current pulses have been plotted for a small number of motoneurones. The time constants derived from these curves lay in the range reported for cat motoneurones (Frank & Fuortes, 1956; Coombs, Curtis & Eccles, 1959).

The accommodation curves of motoneurones

When a current rising linearly to sufficient strength was applied repeatedly to a motoneurone, the threshold at which an impulse was discharged fluctuated widely. Therefore to obtain a measure of the degree

and approximate time course of the accommodation process of the cell, 'triangular' current pulses of 5–7 different gradients were delivered repeatedly and in random order at 2 sec intervals to the impaled motoneurone via the recording micropipette, and the mean latency and current strength at which the first spike occurred was determined for each current gradient. Samples of records obtained by intracellular stimulation with linearly rising currents are given in Fig. 2. From records such as those of Fig. 2 it was easy to measure the interval from the start of the triangular current pulse to the moment of origin of the spike, i.e the latency. The strength of the current at that moment was read off and each filled circle of Fig. 3 represents the average of 4–12 observations of latency and strength with a fixed rate of current rise. This rate is shown by the slope of the straight line drawn through each point, for that line produced passes

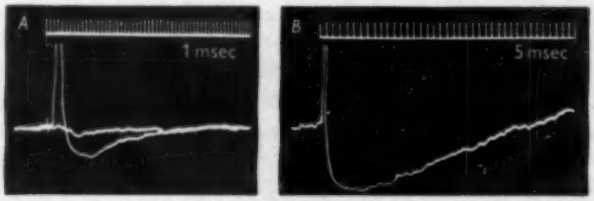

Fig. 1. Comparison of positive after-potentials (after-hyperpolarization) of a motoneurone of a rat and of a cat. *A*: superimposed records of the response to a just subliminal and a just liminal stimulus to the ventral root of a rat. *B*: just liminal stimulus to the ventral root of a cat (recorded at higher gain than *A*).

through the origin (strength zero, latency zero). Individual observations showed some variation, but all lay upon the short segment of line shown.

In most motoneurones it was found that the accommodation process was limited both in time and extent. After the initial descending limb the curve comes to a minimum value at 8–20 msec latency, then rises at a variable slope to a variable degree to reach an apparent ceiling at 40–80 msec latency, after which little or no increase in threshold occurs. The term 'ceiling' is used in this report to mean the current value to which the curve tends at long intervals, expressed in absolute units or as a multiple of the rheobase. The slope of the ascending part of the curve is loosely correlated with the ceiling of the threshold rise, as may be seen in Table 1. Only values obtained from rat motoneurones have been included in Table 1 for the sake of homogeneity of material, but the curves of cat motoneurones had very similar shapes. The large scatter of values may be partly due to the inaccuracy of the method, because owing to the constant fluctuation of excitability of the cells the measurements of both the rheobase and the slope are only approximations. Related to the slope of threshold rise was

the utilization time to rheobasic d.c. pulses (Hauptnutzzeit): if the initial accommodation had a slope of 1/10 to 1/40 I_{rh}/msec the Hauptnutzzeit was never greater than 3 msec; with slopes less steep than 1/200 it lay between 5 and 30 msec and had a large variability in the same cell.

An interesting and unexpected relationship was found when the cells were grouped according to the apparent ceiling of accommodation and

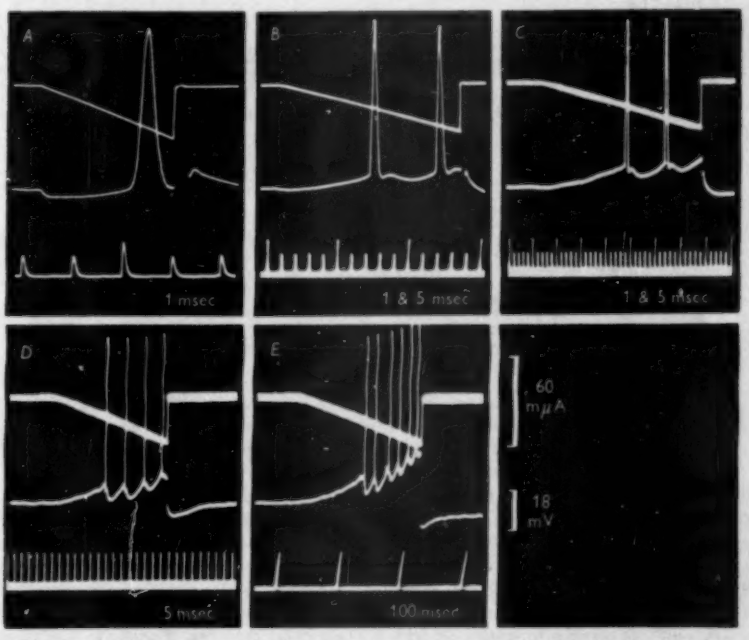

Fig. 2. Oscilloscope records from a rat motoneurone stimulated with linearly rising currents. Upper trace: stimulating current pulse, downward deflexion represents outward current. Upper vertical bar, representing 60×10^{-9}A, refers to intensity of current. Lower trace: potential at micro-electrode tip, positivity upward. Voltage calibration (18 mV): lower vertical bar. Note different time scales for each record.

their various properties compared. In Fig. 4 the ceiling of accommodation is plotted against the measured rheobase (I_{rh}) of the same cell. There is no true correlation between these two values, but neither are they completely dissociated, rather the points tend to cluster in two discontinuous groups: in cells with a high ceiling the rheobase was always less than 4 nA, in cells with a ceiling less than 1·5 I_{rh} the rheobases were distributed along the whole range observed. The mean I_{rh} for 'high-ceiling' cells was only 1·9 nA, for 'low-ceiling' cells 7·7 nA (Table 2). It should be

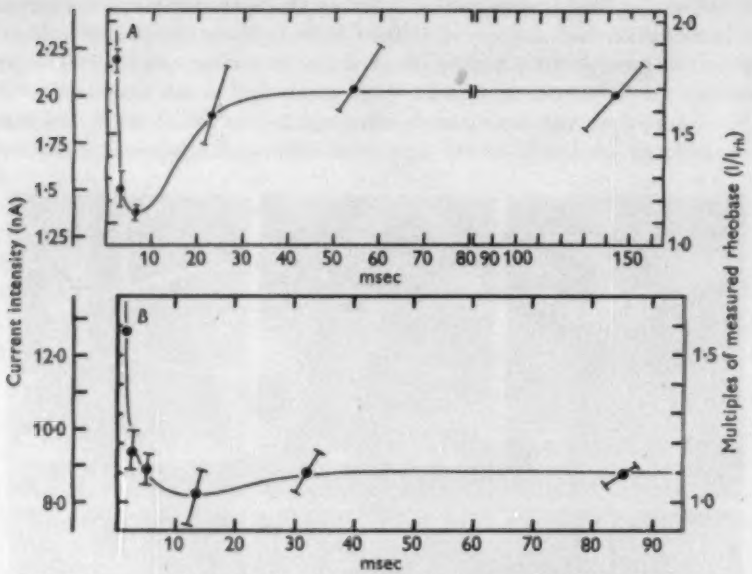

Fig. 3. Accommodation curves, of two rat motoneurones. *A*: 'high-ceiling' type of accommodation. *B*: 'low-ceiling' type of accommodation. Points represent average threshold current strength (ordinates) and latency (abscissae) of spike discharge to a linearly rising stimulating current. The short straight lines intersecting the points represent part of the time course of the stimulating current, and indicate the limits between which excitation occurred in repeated trials with the same current gradient.

TABLE 1. Distribution of rat motoneurones according to the slope of accommodative threshold increase and the apparent limiting value or 'ceiling' of accommodation as found for individual cells stimulated directly with linearly rising currents. Figures in parentheses indicate the numbers of cells where the measured values of the slope of accommodation and its ceiling fell within the limits given for the corresponding column and row. Examples of curves on which the measurements were carried out are given in Fig. 3. Only rat cells where estimates were reasonably accurate have been included

'Ceiling' of accommodation (I/I_{rh})	Slope of threshold rise (I_{rh}/msec)					
	−1/50	−1/100	−1/200	−1/400	−1/800	1/800−
2·0−	(2)	(1)	—	—	—	—
−2·0	—	(1)	—	—	—	—
−1·9	(1)	—	—	—	—	—
−1·8	(1)	—	—	—	—	—
−1·7	(1)	—	(1)	—	—	—
−1·6	(1)	—	(1)	—	—	—
−1·5	—	—	—	—	—	—
−1·4	—	—	(1)	—	—	—
−1·3	—	—	—	—	—	—
−1·2	—	—	(2)	(1)	(2)	(2)
−1·1	—	—	(2)	(1)	—	(2)

noted that the association of ceiling versus accommodative slope shown in Table 1 also suggests a discontinuous distribution. It is of course possible that with a sufficiently large series intermediate values would also have been found.

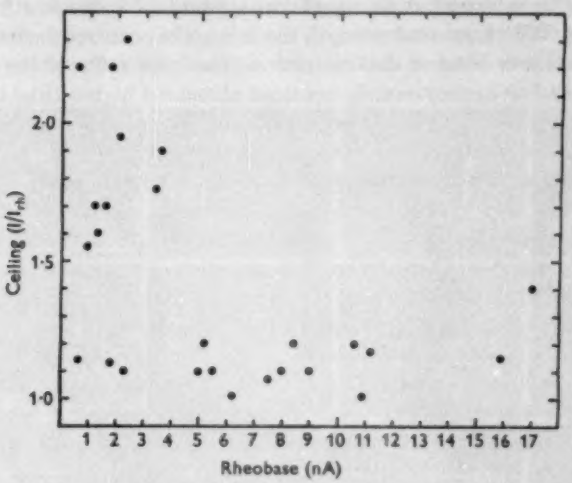

Fig. 4. Relationship of the 'ceiling' of accommodative threshold rise to the measured rheobase of the same cell.

TABLE 2. Mean rheobase of rat motoneurones showing various types of accommodation curves. 'Low ceiling' of accommodation: less than $1{\cdot}5 \times I_{rh}$. 'High ceiling' of accommodation: more than $1{\cdot}5 \times I_{rh}$. 'Rapid m.c.g.' type: apparent λ less than 30 msec. 'Moderate m.c.g.' type: λ more than 30 msec. *n*: number of cells in category indicated. I_{rh}: rheobase in 10^{-9}A. Difference: difference of mean I_{rh}. S.E.: Standard error of difference. Common mean: Mean I_{rh} of all 'ceiling' type and all 'm.c.g.' type cells respectively

	'Ceiling' type		'M.c.g.' type	
	Low	High	Rapid	Moderate
n	21	10	12	9
Mean I_{rh}	7·7	1·92	6·2	5·3
S.D.	±4·35	±1·02	±1·3	±3·13
Difference	5·78		0·9	
S.E.	±1·0		±1·22	
Common mean	5·85		5·8	
Common S.D.	±4·37		±2·17	

The study of the late time course of accommodation was limited because the stimulating circuit could not generate pulses with a rise time longer than 220 msec.

When a stimulating current of constant intensity and sufficient strength was applied to most motoneurones the cells responded with a maintained

repetitive discharge. The discharge of a rat motoneurone to rectangular currents of about 40 msec duration and graded intensity is illustrated in Fig. 5*A–F*. In the records *A* and *B* the stimulus was just liminal, and in *A* no spike was discharged. The regularly recurring small depolarization in *A* may be interpreted as repetitive subliminal responses (oscillatory behaviour). With increased strength the first spike occurred earlier and took off from a lower level of depolarization. The later spike of the repetitive discharge had an approximately constant threshold higher than that of the first spike, i.e. arising from a lower membrane potential, and each seemed to arise when the positive after-potential of the previous spike had subsided to this constant level (see also Kolmodin & Skoglund, 1958). This is also shown in Fig. 5*G*, where the discharge had been maintained with nearly perfect regularity for 1½ sec. In some rat cells, however, the firing rate did not remain constant, but declined slowly during the flow of current, and Eccles (1957) had reported that the repetitive firing of cat motoneurones to a constant depolarizing current often stops after a few seconds. In response to a comparable depolarizing current cat motoneurones fired at a lower frequency than rat motor cells. With the method employed in our experiments the threshold level of membrane potential could not be measured accurately when strong stimulating currents were used, because compensation for the stimulus artifact was inadequate and often also unstable.

Araki & Otani (1959) found no ceiling to the accommodation of motoneurones in the excised cord of toads stimulated with currents of exponentially delayed rise, but the longest latency they recorded was only about 30 msec. The 'LR' type of impulse initiation observed by Araki & Otani (1959) in some toad motoneurones was seen only in one rat motoneurone which had a 'low ceiling' of accommodation. In all other rat and cat motor cells the spike arose abruptly, as shown in Figs. 2 and 5, and a sufficiently fast sweep revealed an inflexion on the rising phase. The variations in the type of accommodation of mammalian motoneurones are therefore not due to differences in the site of impulse initiation.

Anomalous accommodation curves

In a smaller number of motoneurones a well defined minimal current gradient was found and the shape of the accommodation curve corresponded closely to that predicted by Hill (1936), with an apparent value of λ ranging from 7 to 280 msec. Upon Hill's theory the minimum current gradient for excitation is I_0/λ, where I_0 is the rheobase when $\lambda = \infty$. Thus λ is approximately the time this current takes to rise from zero to the actual rheobasic current value. It was initially felt that these curves were obtained from motoneurones of an entirely different class (Somjen &

Bradley, 1959). It was later found, however, that cells of the 'minimal current gradient' (m.c.g.) type could sometimes change readily into the type showing accommodation with ceiling, and that the reverse might also happen, and within the course of observation from a single cell such a change-over might even occur more than once. Figure 6 illustrates the change-over from the 'm.c.g.' type of accommodation to the 'ceiling' type of accommodation in a single cell. In this case the rheobase declined

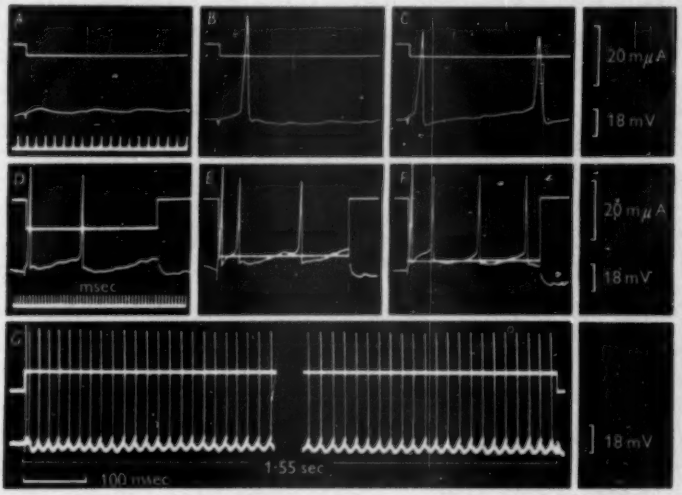

Fig. 5. *A–F*: records from a rat motoneurone, stimulated with a 40 msec rectangular current pulse of varying intensity. Upper beam: stimulating current, downward deflexion indicates outward current. Lower beam: potential at microelectrode tip, positivity upward. *A–C* at faster sweep than *D–F*. Stimulus is at rheobase strength for *A* and *B*, increased strength from *C* to *F*. *G*: another rat motoneurone, stimulated with long (1·5 sec) pulse of which only the beginning and end are reproduced here. The trace bisecting the spikes indicates make (upward deflexion) and break (downward deflexion) of stimulating current, the strength of which was approximately 2 × rheobase ($10{\cdot}8 \times 10^{-9}$A).

steadily during the shift, but this did not occur in all cells. Furthermore, each time the 'm.c.g.' type of accommodation changed into 'ceiling' type, the recorded amplitude of the spike elicited antidromically or orthodromically increased, whereas the reverse change was always associated with a decrease of spike amplitude. The mean spike amplitude of all 'ceiling' type cells was 76·3 mV (S.D. ± 13·2), that of 'm.c.g.' type cells only 58·4 (S.D. ± 10·1). The difference of almost 18 mV is significant (S.E. of the difference of means: ± 3·5). There was no obvious correlation within the 'm.c.g.' type cells between the spike size and the apparent

value of the time constant of accommodation λ. The mean rheobase of the cells of 'm.c.g.' type was about the same as that of all 'ceiling' type of cells, intermediate between the mean rheobase of 'high-ceiling' type and 'low-ceiling' type, and there was no detectable correlation between the rheobase of the 'm.c.g.' type cells and the rapidity of accommodation (Table 2).

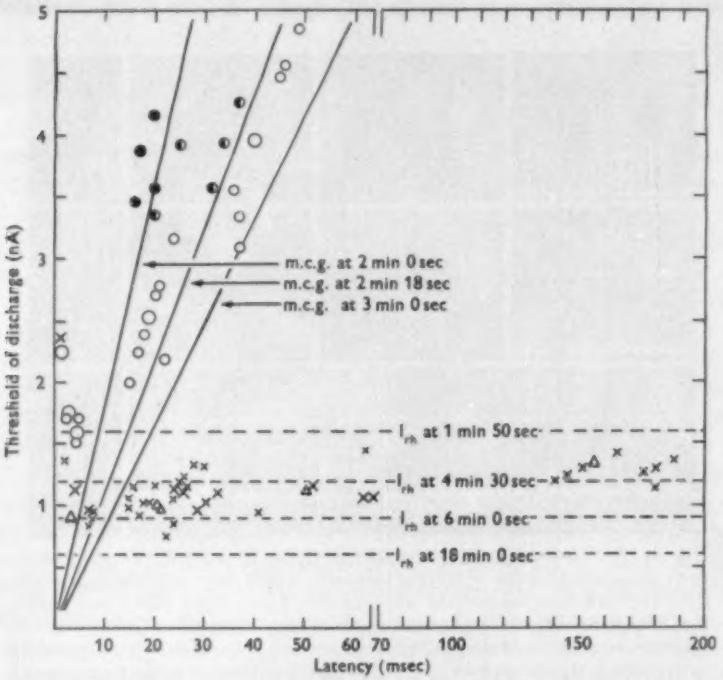

Fig. 6. Change of accommodation observed in a rat motoneurone. ●, experimental points 2 min–2 min 6 sec after impalement of cell; ◐, 2 min 8 sec–2 min 16 sec; ○, 2 min 18 sec–4 min 4 sec; ×, 12½ min–15 min; △, 15½–17 min after impalement. Large symbols = mean of several measurements; small symbols = measurement from individual records. m.c.g., minimal current gradient at time indicated; I_{rh}: level of rheobasic current intensity.

Figure 7 illustrates the relationship of spike size to rheobase in the three classes of cells investigated. It may be seen that there is no over-all correlation between spike size and rheobase, but that all 'm.c.g.' type cells (triangles on figure) had spikes less than 75 mV. The difference in rheobase between 'high-ceiling' (open circles) and 'low-ceiling' (filled circles) cells is also evident, and also that there is little apparent relationship between the ceiling of accommodation and the amplitude of the spike.

The mean spike amplitude of cells in which the ceiling of accommodative threshold rise was below $1{\cdot}5 \times I_{rh}$ was 78·1 mV, and in cells with a higher ceiling of accommodation the mean spike amplitude was 72·7 mV. The difference of means was 5·4, which was less than the standard error of the difference ($\pm$ 6·75). The limited material available included no example where the ceiling of accommodation shifted spontaneously.

It is likely that the cells with 'm.c.g.' type of accommodation are recruited from both the 'high' and 'low-ceiling' types of cells, and that the change-over is associated with, if not caused by, partial depolarization of the cell.

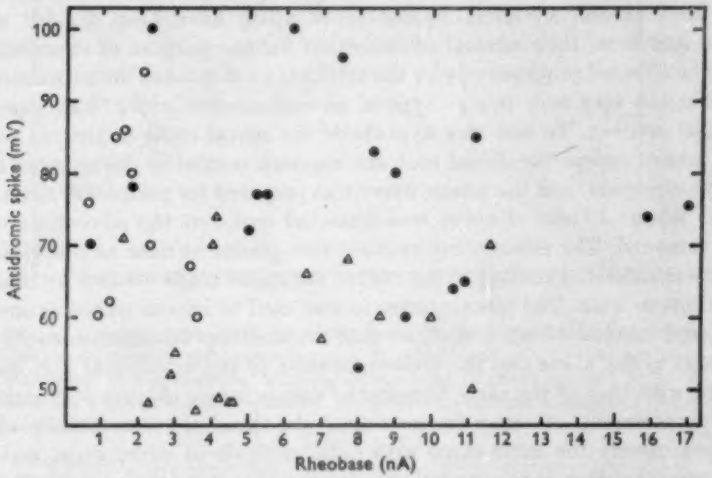

Fig. 7. Relationship between amplitude of antidromic spike and rheobase (I_{rh}) of individual rat motoneurones. ●, cells with 'low-ceiling' type of accommodation; ○, 'high-ceiling'; △, 'm.c.g.' type of accommodation.

Accommodation of motor axons

The accommodation of a motor fibre could be compared with that of its parent cell by applying a stimulus to the ventral root or peripheral nerve and recording the antidromically conducted spike from the cell body. After correction for conduction time the curve of threshold against latency for various gradients of current could be plotted in the same way as has been done for direct stimulation of the motoneurones themselves.

As a rule fibres stimulated in the ventral roots of rats appeared to accommodate very rapidly. In most cases a minimal current gradient with inverse slope (λ) of 0·8–1·5 msec was found, and currents rising more slowly failed to elicit a discharge even if continued up to 15–20 times

rheobasic strength. Ventral root fibres usually responded to strong rectangular stimulating currents with only a single spike. The latency of response to rheobasic currents was about 1 msec or less. The threshold to anodal 'break' was the same as the cathodal rheobase, provided the anodal pulse had a sufficiently long duration (30–200 msec). Essentially similar results were obtained by stimulation of cat ventral roots, but the apparent value of λ was longer (about 4 msec).

In two experiments on rat ventral root fibres immediately after the dissection had been completed, accommodation was much slower than usual, but became more and more rapid as time went on. In one fibre the rate of accommodation appeared to increase by a factor of almost 4 within the span of half an hour. Spinal roots which have been divided and separated from their normal environment for the purpose of stimulation may be affected progressively by the artificial conditions of the experiment, so that the very fast 'm.c.g.' type of accommodation could be an experimental artifact. To test this hypothesis the spinal roots in the rat were left intact, except the dorsal root one segment cranial to the entry of the micro-electrode, and the sciatic nerve was prepared for peripheral stimulation. About 10 mm of nerve was dissected free, but the adventitia was not removed. The stimulating cathode was placed as near as practicable to the undissected portion of the nerve, the anode made contact by means of a cotton wick. The micro-electrode was used to impale motor axons in the cord instead of cell bodies, so that intracellular stimulation could be applied to the fibres and the accommodation of the intraspinal part compared with that of the same fibre in the sciatic nerve. Figure 8 illustrates this experiment. It may be seen that the threshold rose initially with approximately the same slope with both methods of stimulation, but in the sciatic nerve accommodation came rather abruptly to a 'ceiling', whereas for intracellular stimulation in the cord the slope of the threshold decreased more gradually.

When intracellular stimulation of motor axons was applied in rats with severed ventral roots, the accommodation of the intraspinal part of the fibre was found to be as fast as that of the ventral root (three observations only). It appears, therefore, that severed ventral roots are subject to irreversible alteration, detectable by accelerated accommodation and lifting of the 'ceiling' of the accommodative increase in threshold, which on the basis of these few results extends into the intraspinal section of the fibres. Unfortunately it was not possible to determine whether the fibres were penetrated in the white or grey matter. Some uncertainty attaches to the method of intracellular stimulation because impaled fibres are particularly liable to depolarization, and, as in cell bodies, depolarization of the fibre was associated with an increase of accommodation.

In the cat an inflexion on the ascending limb of the accommodation curve was found in some motor fibres stimulated in the popliteal or biceps-semitendinosus nerves, and these fibres generated prolonged repetitive discharges with strong d.c. currents. The tendency for repetitive discharge was increased if the paraffin pool covering the nerve was cooled.

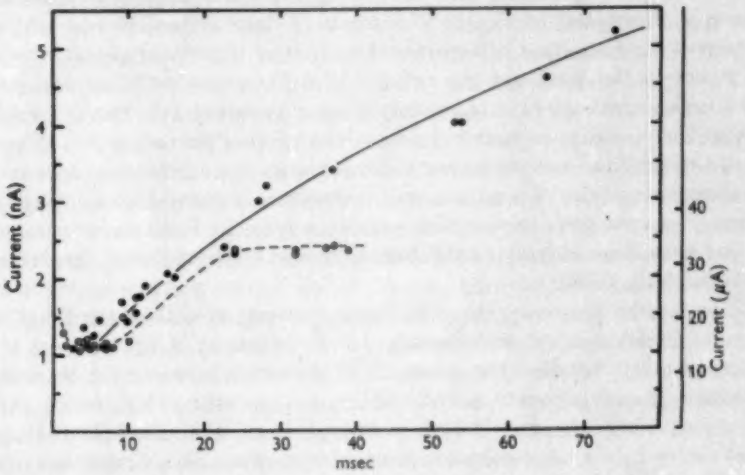

Fig. 8. Accommodation of a rat motor axon. ●, and continuous line: intracellular stimulation with microelectrode in spinal cord. ○ and broken line: stimulation of sciatic nerve, recording intracellular. Current strength in 10^{-9}A for intracellular (left-hand ordinate), 10^{-4}A for extracellular stimulation (right-hand ordinate).

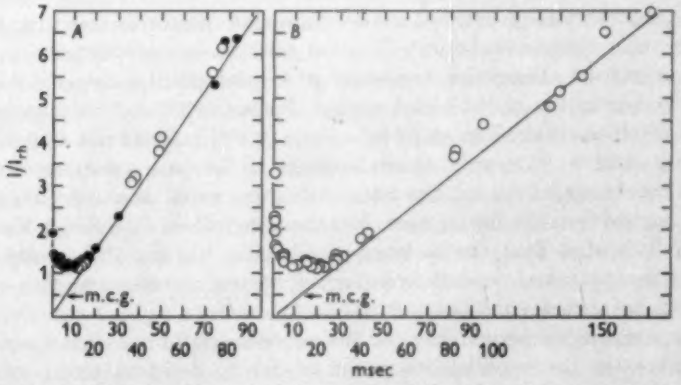

Fig. 9. The effect of temperature on the accommodation of a cat's motor nerve fibre stimulated in the popliteal nerve. *A*: at 37° C; ●, before cooling; ○, after cooling and rewarming. *B*: at 30·5° C.

In other fibres a definite minimal current gradient appeared to be present under the same conditions, but the currents applied to cats' peripheral nerves did not exceed $7 \times I_{rh}$, and a ceiling of accommodation might have existed at a higher level. The minimal current gradient was sensitive to variations of temperature. The change of accommodation observed in the same fibre during cooling and rewarming the stimulated section of the nerve is illustrated in Fig. 9. The mean of four determinations of the apparent time constant of accommodation at or slightly above 37° C was 13 msec; in the same cat the mean λ of 6 fibres at 30·5° C or less was 40·6 msec, which gives a Q_{10} value of approximately 4·5. The Q_{10} value derived in a similar experiment for rat ventral root fibres was 3·1. Whenever a motor fibre was cooled the value of the measured rheobase appeared to decrease and the time constant of excitation (k) derived from strength–latency curves with rectangular pulses increased. Both these changes might have been at least partly due to the slowing of the accommodation process (Hill, 1936).

The threshold–latency curve of Fig. 9*A* closely resembles the function derived for stimulation with linearly rising currents by Hill (1936, eqn. 24), except for the fact that the minimum of the curve is nearer the observed rheobase than expected from Hill's theory for this ratio of λ/k. In Fig. 9*B*, however, where accommodation is slowed down, the threshold remains just above I_{rh} for a considerable range of latencies, and a similar flat part at the minimum value may be seen in the curve of the rat sciatic nerve fibre represented by the broken line in Fig. 8. The significance of this deviation from Hill's (1936) theoretical function will be discussed later.

DISCUSSION

The most striking feature of the accommodation process emerging from the present work is its lability. Various authors have reported that the accommodation of nerves is increased after mechanical damage (Solandt, 1936), interruption of the blood supply (Liesse, 1938) and by catelectrotonus (Shanes, 1940; Tasaki, 1950). Katz (1939) pointed out that if the accommodation of a preparation appears to become more rapid it is probably wrong to regard the later stationary rapid accommodation as more normal than the initial, slow, but changing value. Hodgkin & Huxley (1952) indicated that the isolated squid axon has an abnormally low membrane potential, and therefore may appear to accommodate more than under normal conditions.

The irreversible acceleration of the accommodation of spinal ventral root fibres in our experiments might be due to deficient blood supply, because arterial blood normally flows into the spinal cord along the roots and these had been severed just inside the intervertebral foramen, whereas

the blood supply of the peripheral nerves may have been better preserved. The often reversible changes of the degree of accommodation of impaled cells and fibres found with intracellular stimulation are probably due to depolarization caused by mechanical damage, which may later be reduced by 'sealing in' of the micropipette's tip, or may vary because of small movements of the preparation.

The method used in this study does not furnish information about the early time course of accommodation, because the accommodative rise of threshold at short latencies is obscured by the initial descending limb of the curves obtained with linearly rising currents. These curves may be regarded as being built up from two separate components: a descending part analogous to the well-known strength–duration curves obtained with rectangular current pulses or condenser discharges, and a rising phase which represents accommodation. An attempt to analyse the experimental curves into these two elements and so obtain an approximate extrapolation of the 'true accommodation curve' to zero time is presented separately (Somjen, 1961). The proper experimental approach would be to measure the level of displacement of membrane potential instead of the level which the current had reached at the instant of excitation. Unfortunately with the bridge circuit employed in our experiments the displacement of membrane potential was usually distorted by the stimulus artifact, which often changed its shape during the course of observation.

Although our findings do not permit any definite conclusion about the nature of the accommodative change of threshold it seems worth while to enquire whether they are compatible with theoretical suggestions of other workers. Our experimental curves depart in two respects from the function derived by Hill (1936) to describe the conditions for excitation with linearly rising currents. In agreement with the results of Skoglund (1942) on cat's sensory and motor nerve fibres, we could not define a minimal current gradient for the stimulation of mammalian motoneurones. Another discrepancy between Hill's theoretical function and some of our results is the flat section that occurs in the minima of some of our curves (Figs. 8 and 9*B*), indicating a range of latencies where the threshold to linearly rising currents remains at or near the rheobase value. Tasaki (1950) has made a similar observation on isolated nodes of Ranvier of toad nerve fibres. This indicates a virtual delay of the development of accommodation. Even for curves which have no obvious flattening at the minimum, the minimum of the curve falls at a lower value than that calculated with the aid of a fixed rate constant of threshold rise, but a satisfactory empirical equation could be fitted to our experimental curves when it was assumed that the growth of accommodation is governed by a variable instead of a constant (Somjen, 1961).

A physical basis for accommodation has been suggested by Hodgkin & Huxley (1952), who found that the ability of the squid giant axon membrane to increase its permeability to sodium ions is impaired if a subthreshold depolarization is imposed on it ('inactivation') and that concurrently the permeability to potassium ions increases, both changes tending to increase the threshold for excitation. Hodgkin & Huxley (1952) found that the Q_{10} value for all their rate constants was approximately 3, which is, considering the species difference, in good agreement with the Q_{10} value of accommodation reported here for mammalian nerve fibres. Qualitatively Hodgkin & Huxley's ionic theory allows for the apparent delay of the increase of threshold evoked by linearly rising currents, because their rate constants are dependent on the level of membrane potential which is in the case of linearly rising currents a function of time. However, Hodgkin & Huxley's theory as it stands cannot account for the apparent ceiling of the accommodative threshold rise in response to linearly rising currents; according to their data the steady-state ionic membrane current at any level of depolarization is outwards, and it follows that if the membrane potential declines sufficiently slowly a self-regenerative response can never occur (see Hodgkin & Huxley, 1952). Part of the difficulty is removed by the conclusion of Eccles (1957) that in cat spinal motoneurones the permeability to K is not increased by any depolarization too small to cause actual excitation. This leaves 'inactivation' as the only factor to explain accommodation. There are no experimental data available from which the various parameters of 'inactivation' in mammalian motoneurones could be deduced, so that it is not possible to calculate whether it would permit excitation to occur even with very gradual depolarization.

Some indirect evidence to support the proposition that accommodation is related to an impairment of the sodium permeability mechanism can be derived from the observation that ether increases the accommodation of peripheral nerve (Heinbecker & Bartley, 1940) and of motoneurones (Somjen & Bradley, 1959). This drug raises the threshold of motoneurones without change of membrane potential (Somjen & Gill, 1958) and therefore it probably is a 'membrane stabilizer' (Shanes, 1958) so that its threshold-raising effect might be expected to summate with that of 'inactivation'.

Our finding that it is possible to excite motoneurones with depolarizing currents rising very slowly, and that rhythmical firing can be maintained by an electrical stimulus of constant intensity is compatible with the proposition that 'central excitatory state', facilitation, and rhythmical reflex discharge are due to sustained synaptic depolarization.

SUMMARY

1. Electrical records obtained by means of Ling–Gerard micropipettes from rats' and cats' motoneurones have been compared. The only significant difference found was the shorter duration of the positive after-potential (after-hyperpolarization) of action potentials from rat motoneurones, and this corresponds to their higher rate of firing in response to d.c. stimuli or tetanization of the dorsal root.

2. The accommodation of rat and cat motoneurones has been investigated by passing linearly rising current pulses directly through the membrane of single cells and observing the threshold for discharge of impulses, or by passing a suprathreshold d.c. current and observing the repetitive firing so generated.

3. Usually accommodation appeared to occur only to a limited extent and was almost complete in the initial 40–80 msec of the flow of current. The slope of the increase of threshold to linearly rising currents was related to the ceiling to which the threshold tended. A minimal current gradient could not usually be defined. A high ceiling of accommodation was associated with a low rheobase. The ceiling of accommodation varied from less than 1·1 to more than 2·0 times the measured rheobase.

4. In a smaller number of observations a definite minimal current gradient was found. In these cells the mean amplitude of the orthodromically and antidromically elicited spike was significantly smaller than in the cells where accommodation appeared to be limited by a ceiling. A few cells were observed to change from 'minimal current gradient' type to 'ceiling' type; this change was always associated with a growth of spike amplitude, and the reverse change with a decrease of spike amplitude. It is concluded that the accommodation process continued without limit only in motoneurones in a state of partial depolarization.

5. In motor fibres stimulated in severed ventral roots, accommodation appeared to be much slower in fresh preparations than later in the same experiment; motor fibres stimulated in peripheral nerve were more stable in this respect. Lowering of the temperature of the paraffin pool slowed down the accommodation of motor axons considerably; the Q_{10} value of the slope of the accommodation curve was of the order of 3–4·5. Changes due to variation of temperature were completely reversible.

We are indebted to Professor A. K. McIntyre for suggesting the subject of this investigation, and encouragement throughout the work and to Professor J. R. Robinson for criticism of this manuscript. Our thanks are due to Dr F. F. Foldes of the University of Pittsburgh, Department of Anaesthesia, for the gift of C-Toxiferin. Most of the electronic equipment used in this investigation was designed, built and serviced by Mr E. A. Annand of the Medical Research Council of New Zealand. Part of the cost was defrayed by the Medical Research Council of New Zealand.

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Note added in proof. Two papers on this subject arrived after the manuscript of this article had been despatched, and therefore have not been discussed. They are:

FRANK, K. & FUORTES, M. G. F. (1960). Accommodation of mammalian motoneurones of cats. *Arch. ital. Biol.* **98**, 165–170.

KOIZUMI, J., USHIYAMA, J. & BROOKS, C. McC. (1960). Effect of hypothermia on excitability of spinal neurones. *J. Neurophysiol.* **23**, 421–431.

J. Physiol. (1961), **156**, *pp.* 93–111
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ACID SECRETION BY THE ABOMASUM AND ITS RELATION TO THE FLOW OF FOOD MATERIAL IN THE SHEEP

By R. W. ASH

From the Rowett Research Institute, Bucksburn, Aberdeen

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The abomasum or fourth chamber of the compound stomach of ruminants is analogous to the simple stomach of monogastric animals. It is situated between the omasum and duodenum and consists of a fundic and a pyloric region which are histologically similar to those of the simple stomach.

There is a continuous secretion of hydrochloric acid by the fundic region of the abomasum during normal feeding but there is still little information available on the patterns of secretion which may occur, or on the factors responsible for maintaining the secretion. Indirect evidence for the flow of food material from the forestomach into the abomasum being a major stimulus to acid secretion was provided by Hill (1955).

The aims of the experiments reported in the present paper were, first, to observe the secretory responses of innervated fundic pouches during feeding, fasting and rumination; secondly, to relate the responses to the conditions existing in, and the flow of food material through, the abomasum. The results obtained suggested that under normal conditions the inflow of predigested food from the forestomach, the secretion of acid by, and the flow of material from the abomasum were integrated. Further, the acidity of the abomasal contents was an important factor regulating the secretion of acid.

METHODS

Surgical preparation

The sheep used in the experiments were kept indoors for a minimum of 4 weeks before any surgical preparation began. All the operations were performed aseptically and under pentobarbitone anaesthesia; during the pouch operations the sheep received an intravenous infusion of glucose-saline.

The first operation was the insertion of an ebonite cannula, diameter 2 cm, into the dorsal sac of the rumen. After an interval of not less than 1 month innervated fundic pouches were made. The method employed was fundamentally that described by Hill & Gregory (1951) with certain modifications. Briefly, these included emptying and rinsing the reticulorumen the night before the operation and returning 3–4 l. of isotonic NaCl solution to the rumen overnight; the saline was drained from the rumen immediately before the sheep was anaesthetized. This procedure helped in obtaining a fairly clean abomasum at the operation.

The pouch was constructed from the caudal end of the fundic part of the abomasum and the transection was made 2–3 cm cranial to the junction of the pyloric antrum with the fundic region. The interior of the organ was rinsed clean and examined for the presence of helminths or other lesions. *Haemonchus contortus* worms were removed when present. When making the septum between the fundus and the pouch no attempt was made to resect the mucous membrane covering the large mucosal folds which run downward and backward from the omaso-abomasal orifice. Instead, each fold was cut completely down to its base and then resected for 10–15 mm on both sides of the incision; the cut ends of the folds were not sutured. After suturing the internal ring of mucosa, closing the main body of the abomasum, the exposed submucosal tissues were approximated with a continuous suture. The anastomosis of the pyloric antrum to the main body was made close to the septum. An ebonite cannula, diameter 1 cm, was inserted into the fundus at a point opposite the anastomosis and a silver or Perspex cannula used to drain the pouch. At the end of the operation all the pouches were oedematous.

As soon as the sheep was able to stand, 3–5 hr after the operation, 3–4 l. of rumen fluid from normally fed sheep was introduced into the rumen. During the following 48–72 hr the body of the abomasum was drained at 4–8 hr intervals; this reduced the possibility of breakdown of the septum from excessive distension. The pouches were rinsed two or three times daily with isotonic NaCl solution until haemorrhage stopped, usually by the fourth post-operative day. Many of the sheep began to eat after they recovered from the anaesthetic, but then refused food for the next 2–3 days. A complete return of appetite took up to 14 days. Six operations on adult sheep were completely successful out of nine attempts and one further success was achieved on a lamb operated on when 4 weeks old. The simple preparation is shown diagrammatically in Fig. 1(*a*).

Three sheep previously prepared with fundic pouches were fitted with pyloro-duodenal re-entrant fistulae, the operations being performed 2, 3 and 8 months after preparing the pouches. The duodenum was transected at its junction with the pylorus and care was taken to avoid damaging the hepatoduodenal branch of the vagus nerve and the duodenal and abomasal blood vessels; all duodenal tissue was trimmed from the pyloric stump. The ends of the duodenum and the pylorus were closed with an inversion suture and cannulae with gutter-type internal flanges and curved barrels (Phillipson, 1952) inserted. Perspex cannulae were used initially but they were not suitable. Usually the abomasum and duodenum of the sheep are quite mobile, but the preparation of the pouch with the subsequent formation of adhesions anchored them. As a result it was difficult to position the Perspex cannulae, and being non-pliable they caused pressure necrosis of the mucous membranes. This was overcome by moulding cannulae from a pliable material (Dunlop DS/800-V, Dunlop Rubber Co. Ltd.). The important features of the cannulae were that the curved barrels, bore 1·2 cm, joined the internal flanges at 45° and the flanges were quite soft. The cannulae were exteriorized through stab incisions and the continuity of the flow restored with a Perspex or glass junction piece. The sheep recovered rapidly from the operation and the only hazard encountered was an occasional blockage of the cannulae with food particles or wool. The complete preparation is shown in Fig. 1(*b*).

Maintenance of animals

The sheep were kept in individual pens or metabolism cages; food and water consumption was recorded daily. Unless it was otherwise desired, the animals were fed twice daily either at 7 a.m. and 7 p.m. or 9 a.m. and 4 p.m. The amount of food allowed daily was usually 750 g, which was less than they consumed when fed *ad libitum*. This amount was consumed by all the sheep regularly, maintained their body weight and provided a convenient base line of acid secretion on which to superimpose the effects of changes in diet. Free access to water and a block of NaCl was allowed. Those sheep which secreted large volumes of juice from their pouches received an additional 5–10 g NaCl per day sprinkled on the food. No attempt was made to replace hydrogen ion since it was found that an infusion of artificial secretion

(total acid 80–115 m-equiv/l.) into the abomasum caused a profound inhibition of acid secretion by the pouch. Samples of jugular blood were taken from some of the sheep at intervals and the plasma concentrations of Na^+, K^+, Cl^- and total CO_2 were always within the accepted normal range.

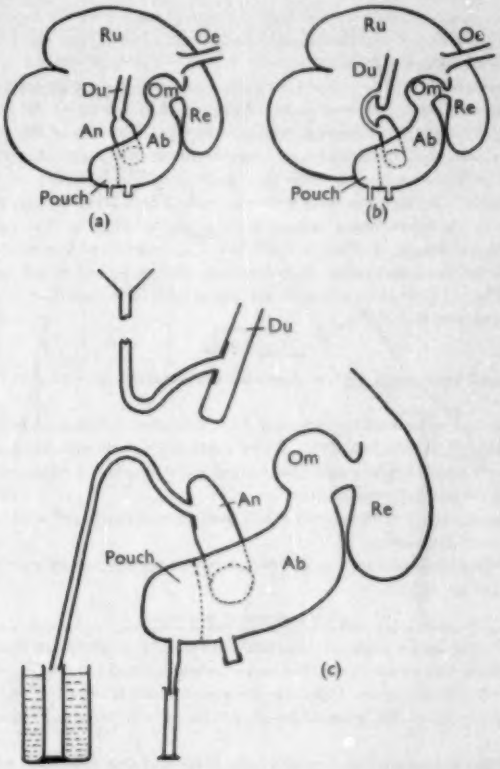

Fig. 1. Diagrams of the preparations used in the experiments. (*a*) Hill–Gregory innervated abomasal pouch with a cannulated abomasal fistula; (*b*) innervated pouch, cannulated abomasal fistula and pyloro-duodenal re-entrant fistulae; (*c*) arrangement used to measure outflow from the abomasum and acid secretion simultaneously (for details see text). All the preparations were fitted with rumen cannulae. Oe: oesophagus. Ru: rumen. Re: reticulum. Om: omasum. Ab: abomasum. Du: duodenum. An: pyloric part of abomasum.

Collection and sampling

Secretions from the pouch were collected by attaching a polythene tube to the pouch cannula and directing the flow into a Pyrex glass cylinder. Free drainage from the pouches was not always achieved, because the large mucosal folds inside them occasionally blocked the internal orifices of the cannulae. At the end of each 15, 30 or 60 min collection period the pouches were drained by gently inserting a smooth glass rod. This procedure did not

appear to affect the results and was adopted in every experiment. Continuous collections of secretion over 24 hr periods for many weeks were obtained by keeping the sheep in a metabolism cage. The polythene tube attached to the cannula was suspended from a light spring and additional support to the cannula was provided by an external flange held close to the abdominal wall by a webbing belt.

Samples of abomasal contents were obtained at intervals during the course of some experiments by opening the cap of the abomasal cannula and allowing not more than 30–40 ml. of the contents to flow into a beaker.

The flow of material from the pyloric cannula was measured by directing it into a graduated cylinder surrounded by a water jacket maintained at 40–42° C. An attempt was made to measure every 30–50 ml. of material, to retain 10% and introduce the remainder into the duodenum. However, this was not always possible, because at high rates of flow two or more gushes occurred in rapid succession with the result that up to 100 ml. of material accumulated in the cylinder. The samples retained were bulked into 30 or 60 min collection periods. The importance of re-introducing material into the duodenum has been discussed by Phillipson (1952) and Hogan & Phillipson (1960). The validity of the method in the present experiments will be discussed later. Simultaneous collections of pouch secretion and abomasal outflow (Fig. 1(c)) were made with the sheep held in a recording stand; the duration of the experiments was 6–8 hr.

Analytical

pH measurements were made with a glass-calomel electrode system and Pye direct reading meter.

Titratable acid was estimated by titrating 1 ml. portions of the secretion to the end-point of phenolphthalein with 0·05 N-NaOH. After centrifuging the abomasal contents 5–10 ml. of the supernatant fluid was titrated electrometrically to pH 7·0 with 0·1 N-NaOH.

Total CO_2 was estimated manometrically.

Steam volatile fatty acids in the supernatant fluid from centrifuged abomasal samples were estimated by steam distillation.

Dry matter in the abomasal samples was estimated by drying portions of the whole contents to constant weight at 105° C.

Post-mortem examination

At the end of their useful experimental life the sheep were shot and the relevant parts of the gastro-intestinal tract examined. The most common reason for killing the sheep was loss of one or other of the cannulae. Only one animal showed necrosis of the mucosa and this was caused by pressure of the internal flange of the pyloric cannula; three of the sheep are still alive.

The whole of the abomasum and pouch were dissected free from fat, rinsed and blotted. The mucous membrane was scraped from the pouch, the main body of the abomasum and the pyloric antrum. The size of the pouch was expressed as a percentage of the whole organ (excluding the pyloric antrum) on the basis of the wet weight of mucosa. The size in five of the animals examined ranged from 9·4 to 34%.

RESULTS

Continuous acid secretion

Serial collections at 60 min intervals over 12 hr periods were made on two sheep while allowed to feed *ad libitum*. The output of acid by the pouches showed some variation from hour to hour but there was no consistent effect of feeding. When the sheep were fed twice daily it was usual to observe definite secretory patterns. The volume of the secretion and the

concentration of acid usually showed an increase in the first hour after feeding began and reached a peak in 2–3 hr. Thereafter the output of acid tended to decline, although in some experiments periods of increased output were superimposed on the declining phase (Fig. 2). In other experiments the increase in secretion was delayed until the second hour after feeding (Fig. 5). There appeared to be no consistent effect of rumination on acid secretion in any experiment.

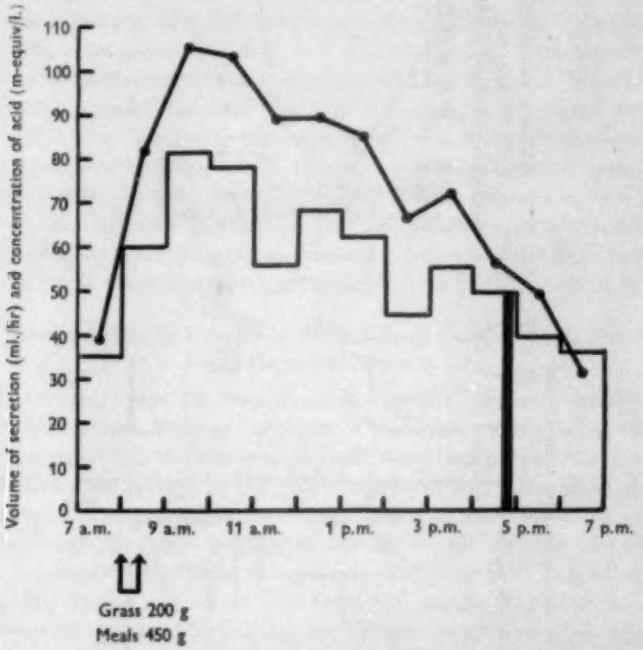

Fig. 2. The secretion of acid by an innervated pouch over 12 hr. The sheep was normally fed with 'meals' 450 g plus chopped dried grass 200 g at 7 a.m. and 7 p.m. Shaded area indicates the time the sheep spent ruminating. Experiment performed 3 months after operation; size of pouch was 31 % of the abomasum. Volume of secretion — : concentration of acid ●—●.

Short-term effects of feeding and rumination

In order to investigate any immediate effects or those with a time course less than 60 min, serial collections were made at 15 min intervals. The amount of food offered in these experiments was 250–450 g and it was all consumed in 10–20 min. To avoid the possibility of conditioning the sheep the experiments were carried out at different times of the day and the animals were allowed to feed 30–90 min after beginning the collection.

About 12–18 hr after the previous feed the output of acid from the pouches was relatively small. When dried grass, meal or a mixture of both was fed to the sheep there was often no appreciable effect during the first 15 min. In the subsequent 45 min the output of acid rapidly increased and reached a peak 45–90 min after feeding began (Fig. 3(*a*)).

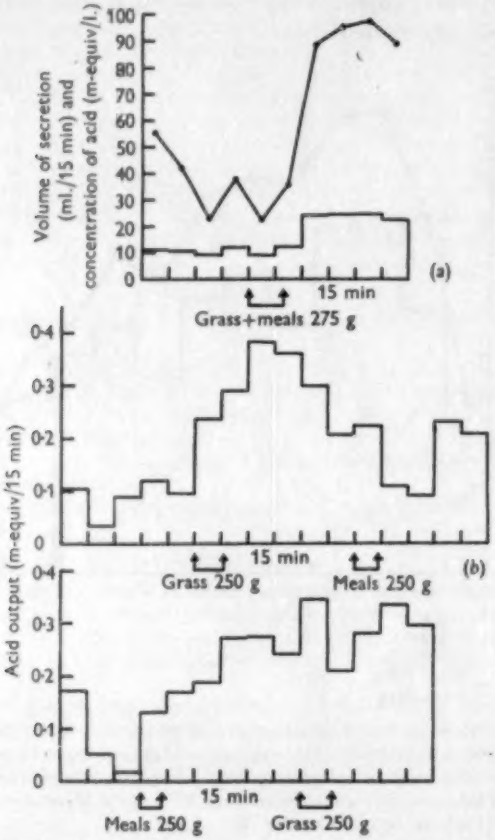

Fig. 3. Some examples of the variation in acid secretion following the feeding of dried grass and meals.

When food was offered close to a previous feeding period and when the output of acid was relatively high the responses to feeding were not consistent. In some experiments the secretion of acid decreased appreciably for 30–45 min and then returned to a level which often equalled or exceeded the pre-feeding period. In other experiments there was no pronounced

effect, while less frequently a small increase was observed. The variability of the responses did not appear to depend on the type of food eaten, although inhibition occurred more frequently when meals were fed to the sheep after dried grass than when dried grass was consumed after a feed of meals (Fig. 3(*b*)). Similarly, the immediate and short-term effects of rumination were inconsistent.

The effect of varying the food intake

The daily output of acid from the pouches of sheep on a standard food intake did show some variation but this was not so pronounced as that observed when the amount of food consumed was changed. When the food intake was increased the output of acid increased; this occurred with dried grass and meals. Reducing the amount of food or depriving the sheep of food reduced the output of acid, although it is important to note that the changes in acid secretion tended to lag behind the changes in diet (Figs. 4, 5 and 6). The amount of water drunk by the sheep on a standard food intake did show some day-to-day variation but when the food intake was changed water consumption usually varied with it (Figs. 5 and 6).

Acid secretion in relation to the acidity of the abomasal contents at the pyloric antrum

The abomasal cannula was situated opposite the anastomosis of the antrum to the main body of the organ. The contents removed at this point had passed through the whole of the acid-secreting part of the abomasum and further acidification of the contents would only take place provided the material remained in the abomasum and acid secretion continued. The pH and total titratable acidity of the 30–40 ml. samples showed only minor fluctuations, but large changes occurred from hour to hour and from day to day in the output of acid from the pouch. The changes in acid secretion were obtained by feeding during the course of an experiment, and altering the food intake between experiments (Fig. 5). As judged by the force at which the contents flowed from the abomasal cannula, the abomasum was distended more when the sheep was on a high food intake than when on the standard diet or when deprived of food.

Similar results were obtained in experiments lasting 6–12 hr on three other sheep. The pH range of the abomasal samples over 12 hr in one animal fed on a diet of hay and flaked maize was 1·6–2·5.

Acid secretion in relation to the outflow of material from the abomasum

Acid secretion by the pouch and outflow from the abomasum were measured simultaneously in two sheep. The experiments were performed

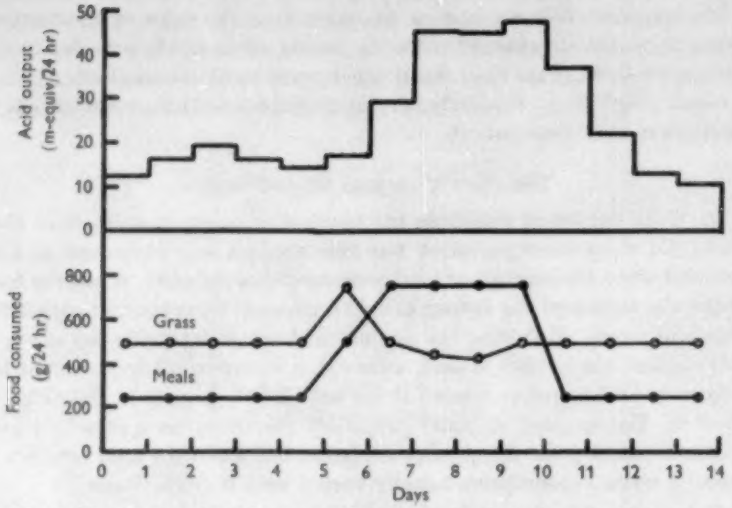

Fig. 4. The effect on the daily output of acid by an innervated pouch of increasing the food intake. Experiment performed 5 months after operation; size of pouch was 9·4 % of the abomasum.

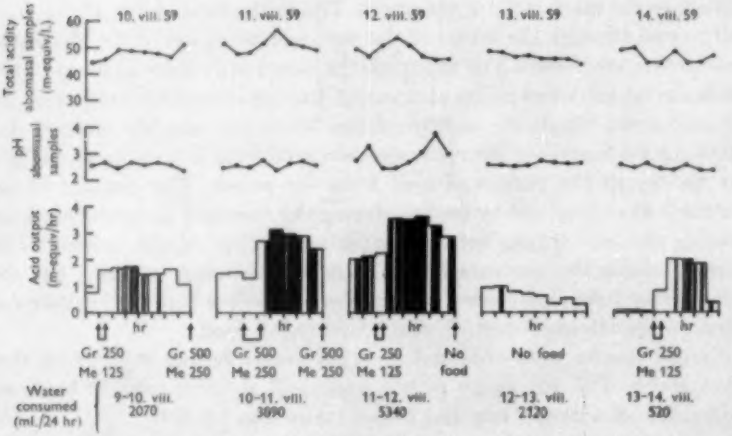

Fig. 5. The small variation in the acidity of the abomasal contents at the pyloric end of the abomasum compared with the large changes in acid secretion by a pouch. Normal diet consisted of dried grass 250 g plus meals 125 g fed at 9 a.m. and 4 p.m. Collection began at 9 a.m. each day and feeding is indicated by the arrows. Shaded areas represent the time the sheep spent ruminating. Gr, dried grass; Me, meals; (weights in grams). Experiment performed 6 weeks after operation.

on 2–5 consecutive days. The flow of material from the abomasum and the secretion of acid by the pouch increased during the first hour after feeding and reached a peak about the second or third hour; thereafter both parameters declined. When the daily food intake was increased, the flow of food material, the amount of water drunk, the secretion of acid and the time spent ruminating all increased while decreasing the food intake caused the opposite effects (Fig. 6). Similar results were obtained from the second

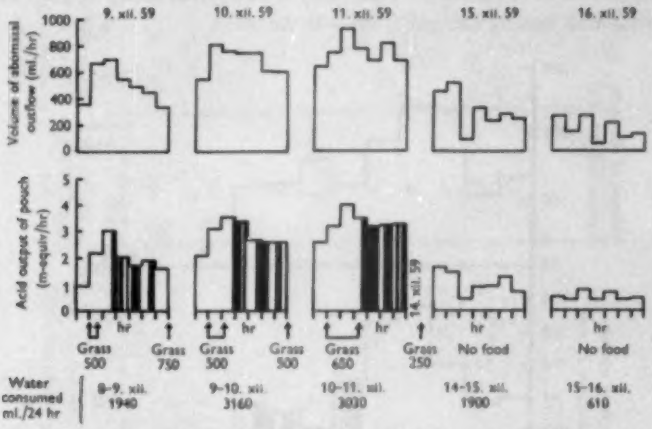

Fig. 6. The effect of increasing and decreasing the food intake (amounts shown in grams) on outflow from the abomasum and acid secretion by a pouch. Normal diet consisted of dried grass 500 g and 250 g fed at 9 a.m. and 4 p.m. respectively. The output of acid and outflow from the abomasum are shown for 60 min collection periods. Simultaneous collections began at 9 a.m. each day and the sheep was fed between the arrows and at the end of each experiment on days 1 and 2. Shaded areas represent the time the sheep spent ruminating. Times between operations and the experiment were pouch 5 months and re-entrant fistulae 2 months.

sheep but the effects of feeding during an experiment were less pronounced when the food intake was increased. A possible explanation was that the sheep did not consume all its ration at once when the food intake was raised but ate at intervals throughout the experiment. Rumination had no consistent effect on the flow of material from the abomasum or on acid secretion. However, on a few occasions when outflow stopped the return coincided with the onset of rumination.

In two experiments the outflow of food material declined to a low level towards the end of the collection periods. On one occasion the animal was fed at that stage but neither acid secretion nor outflow from the abomasum increased (Fig. 7). At the end of the experiment the abomasum was found to be empty.

The maximum rates of outflow observed from both sheep were 931 and 936 ml./hr. The pH values and total titratable acidities of the 30 min collections from both animals remained within narrow limits at all rates of outflow. The linear relationship between the rate of outflow and acid secretion with the pH values and total titratable acidities of the samples from one sheep are shown in Fig. 8. The percentage dry matter of 52 of the samples from one sheep fed on dried grass plus meal was 1·4–4·6 g/100 ml.; the concentration of steam-volatile fatty acid in both sheep under extremes of feeding and fasting ranged from 4 to 30 mM.

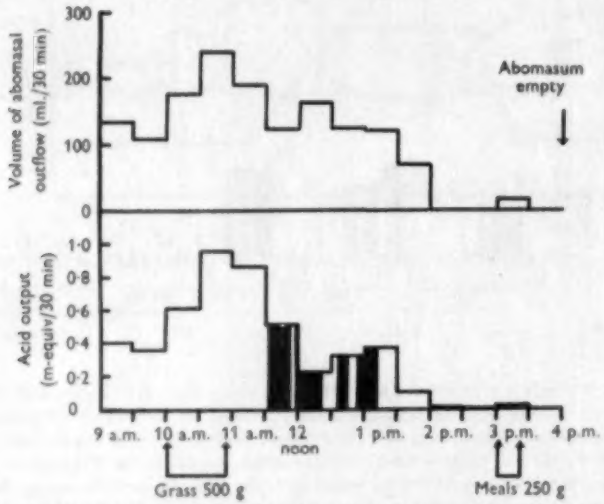

Fig. 7. Simultaneous decline in outflow from the abomasum and acid secretion by a pouch during normal feeding. A second period of feeding failed to re-establish the outflow or acid secretion; it appeared as if the flow of material from the forestomach into the abomasum had stopped. Times between operations and the experiment were pouch 12 months and re-entrant fistulae 4 months.

The effect on acid secretion of preventing and inhibiting the outflow of abomasal contents

Two experiments were performed in which one sheep was previously fed on an increased amount of food to ensure a continuous and relatively high rate of inflow into the abomasum (see Discussion); the sheep was not fed during the experiments. After a suitable period of measuring outflow and acid secretion simultaneously the pyloric cannula was clamped. In both experiments the secretion of acid increased during the subsequent 30–90 min and then declined to a low level (Fig. 9). When the pyloric cannula was opened 150 and 300 min after being clamped, 0·57 and 1·1 l. of

material gushed rapidly from the abomasum; the pH values of these samples were 2·00 and 2·07 respectively.

In two further experiments a condom, length 15 cm, connected to a reservoir of isotonic NaCl solution at 39° C, was inserted through the duodenal cannula and anchored so that the entire length of the condom lay just inside the duodenum. Saline was allowed to run into the condom until

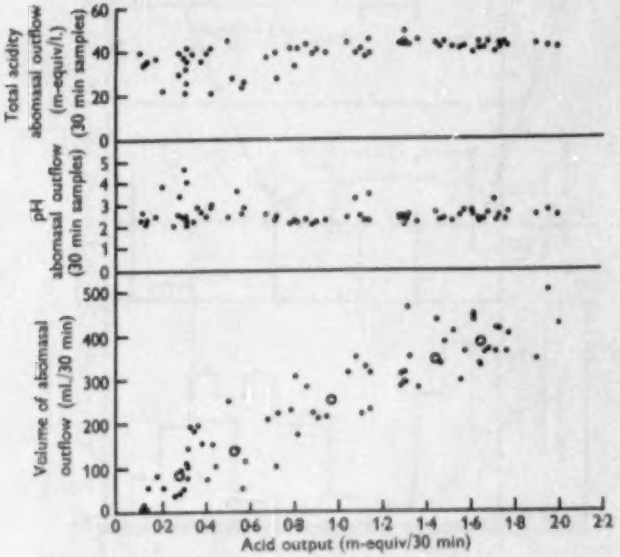

Fig. 8. The relationship between acid secretion by a pouch and the flow of food material from the abomasum. ● 30 min collections; ○ average for each experiment lasting 7 hr. The samples were obtained from the experiments illustrated in Fig. 6.

abomasal outflow was inhibited, the volume required being 50–60 ml. at 40–45 cm of saline pressure. The procedure decreased the secretion of acid in addition to inhibiting abomasal outflow (Fig. 9). When the saline was drained from the condom the secretion of acid and outflow returned simultaneously but there was no rapid and large passage of material from the pyloric cannula.

The effect on acid secretion of infusing acid into the abomasum

Experiments on three sheep showed that when 1 l. volumes of an artificial abomasal secretion, containing 80–115 m-equiv of HCl/l., were infused slowly into the main body of the abomasum, acid secretion by the

pouch was profoundly inhibited (Fig. 10). The secretion collected during the inhibitory period from one sheep contained up to 10·6 mM of CO_2. The samples were not collected anaerobically. An infusion of isotonic NaCl solution did not inhibit the secretion of acid.

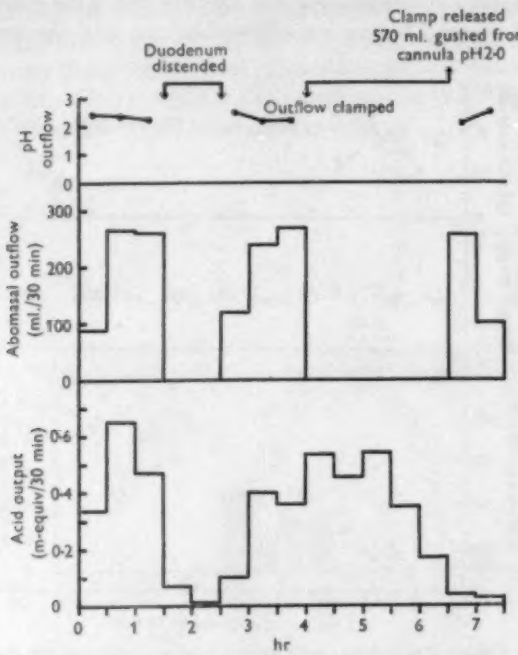

Fig. 9. Comparison of the effects of inhibiting and preventing outflow from the abomasum on the secretion of acid. Inhibition of outflow was caused by distending the duodenum; outflow was prevented by clamping the pyloric cannula. Experiment performed 6 months after the pouch operation and 3 months after preparing the pyloro-duodenal re-entrant fistulae.

The relationship between the rate of flow of secretion and the concentration of acid

In the majority of experiments the concentration of acid in the pouch secretion varied with the rate of secretion (Fig. 11) but the relationship was not absolute. Between experiments ranging over weeks or months on the same sheep relatively high rates of flow were occasionally associated with relatively low concentrations of acid. The reasons for this were not always apparent but the sheep which was found to have a pyloric ulcer developed a high rate of secretion with a relatively low acidity which persisted. The highest concentration of acid obtained from seven sheep during

the feeding regimes adopted was 124 m-equiv/l. At all rates of secretion the relative electrolyte compositions of the samples from all the sheep were similar; these details will be published elsewhere.

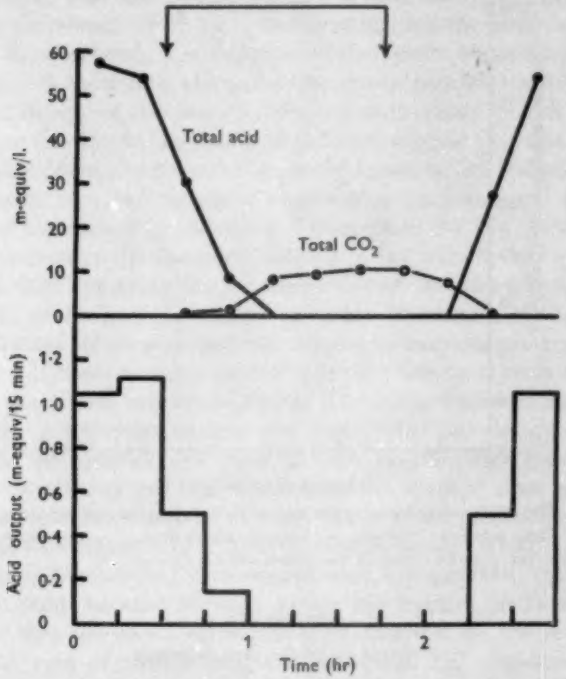

Fig. 10. Inhibition of continuous acid secretion as a result of introducing acid into the abomasum. During the time indicated by the arrows 1 l. of an artificial secretion (total acid 115 m-equiv/l.) was dripped into the main body of the abomasum. Times between operations and the experiment were pouch 4 months and re-entrant fistulae 2 months.

The volume of the abomasal contents

The volume of the abomasal contents in sheep with pouches and pouches plus re-entrant fistulae ranged from 150 to 850 ml. on the standard diets. Five sheep of similar weights and fitted with simple abomasal cannulae were sampled on two occasions while grazing and showed a range of 50–870 ml.

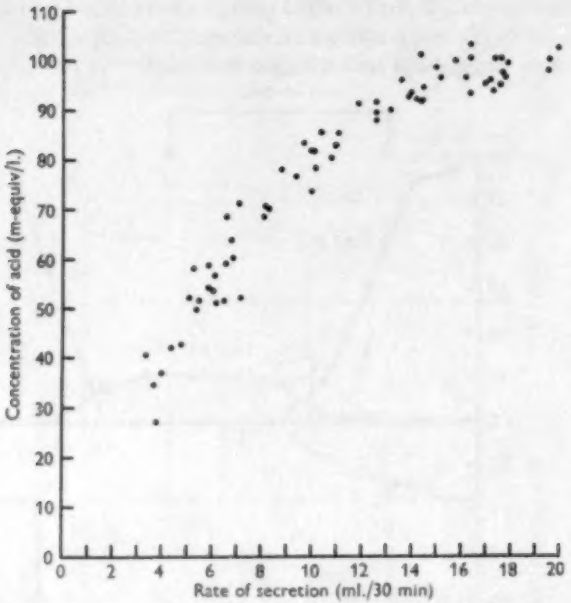

Fig. 11. The relationship between the rate of flow and concentration of acid in the secretion from a pouch. The samples were obtained during the experiments illustrated in Figs. 6 and 8; some of the points are duplicated.

DISCUSSION

Criticisms of the preparations

The validity of the conclusions which are to be drawn from the foregoing experiments depends on whether the acid-secreting cells of the pouch and of the main body of the abomasum respond to physiological stimulation in a similar manner both qualitatively and temporally. The concentrations of acid and the secretory rate per unit weight of mucosa need not be identical; the distribution of acid-secreting cells may not be the same. Experiments on five of the sheep used in the present work suggested that the pouch and fundic region responded similarly to stimuli which were considered to be physiological, e.g. distension of the abomasum and the introduction of rumen fluid and buffered solutions into the abomasum (Ash, 1959*a*). It is possible that the concentrations of acid secreted by the pouch were less than those secreted by the fundic region, since Sommerville (1956) has shown that the number of parietal cells in the abomasal mucosa of the sheep decreased in a craniocaudal direction and the Hill–Gregory pouch used here was made from the caudal end of the abomasum. For this

reason, if the non-acid-secreting cells of the pouch continued to secrete at the same rate as those in the main body of the abomasum a comparatively lower acidity of the pouch secretion is likely.

The effect on secretion and on the flow of food material of reducing the size of the abomasum by 10–30% and exteriorizing the abomasal outflow must also be considered. A comparison of the results from sheep prepared in this way with those from sheep in which the outflow was not exteriorized, and a comparison of the results from the same sheep before and after exteriorizing the flow to the duodenum, do not suggest that this operation caused a significant alteration in the composition of the material leaving the abomasum or on the pattern of secretion by the pouch and main body of the organ (compare Fig. 5 with Fig. 6 from sheep No. 10). In the present work the re-entrant fistulae were inserted in the pyloric antrum and the duodenum, thus bypassing the pyloric sphincter, but the function of this sphincter in controlling the outflow from the abomasum of sheep is not known. Thomas (1957) reviewed the subject in monogastric animals and stated that '...there is as yet no evidence that this function is utilized in regulating the overall rate of emptying.' The method used to measure the outflow can be criticized because the flow of food to the duodenum is temporarily interrupted, and even though the material collected was rapidly re-introduced into the duodenum the method does not mimic exactly the normal course of events. Hunt (1959) considered gastric emptying to be a function of a 'gastroduodenal pump'. The flow patterns must therefore be accepted with reservations, but provided the composition of the food material remains within the normal limits the actual patterns of flow are of no consequence in determining the relationship between the rate of outflow and acid secretion. If a 'gastroduodenal pump' operates in sheep and controls quantitatively the rate at which the abomasum empties then the over-all rate of outflow will be high by 10% because of the amount of ingesta retained for analysis.

The effect of feeding on the secretion of acid

Well defined patterns of acid secretion and abomasal outflow were observed only when restricted feeding regimes were imposed on the animals. It is not suggested that such patterns occur in the grazing animal since the purpose of regulating the food intake in the present experiment was to show what can happen and how it may be produced. Not only was it necessary to regulate the times of feeding but also the amount of each feed so that it was all consumed over a relatively short period. Serial collections made over periods up to 12 hr suggested that diurnal patterns of acid secretion and outflow occurred. These patterns were either less pronounced or apparently absent when the sheep were maintained on the

higher food intakes and also when the food was eaten at intervals throughout an experiment rather than at once.

Acid secretion was stimulated when 250–500 ml. of rumen fluid or buffered solutions was introduced into the abomasum of a sheep with an empty rumen and the responses were greater with the larger infusions; moreover, the responses were potentiated when the concentration of short-chain fatty acids in the solutions was increased (Ash, 1959*a*). The effect of feeding on acid secretion therefore will be influenced by the conditions in the forestomach immediately before feeding, i.e. by the quantity and composition of the forestomach contents capable of being transferred to the abomasum. In the experiment shown in Fig. 7 it seems that food material failed to enter the abomasum during the second period of feeding. It is extremely difficult to know the conditions existing in the forestomach at any one time and to recognize any small changes that may influence the rate of flow of predigested food to the abomasum. Consequently, it is virtually impossible to obtain a true comparison of the effects on acid secretion of feeding either different quantities of the same or a similar amount of different foodstuffs.

The inconsistent responses to feeding and the lag between variation in the amount of food and changes in the acid output, together with the daily variability in acid secretion on a standard diet, indicated that some factor associated with feeding and food intake was more important in determining the amount of acid secreted than feeding and food intake *per se*. The experimental evidence suggested that the continuous and variable patterns of acid secretion were closely related to the flow of food material *through* the abomasum.

Acid secretion in relation to the flow of food through the abomasum

An over-all increase in the secretion of acid by the pouch was accompanied by an over-all increase in outflow from the abomasum, but it should be noted that spurious correlation exists between the parameters in Fig. 8. The material leaving the abomasum consists of the predigested food entering from the forestomach plus the abomasal secretion added to it. As the pH and total titratable acidity of the outflow did not vary very much it can be concluded that an increase in outflow was due partly to a larger flow of material from the forestomach and partly to an increased secretion of acid. The relationship between the rate of secretion and the concentration of acid therein (Fig. 11) precludes the increase in outflow being caused solely by an increase in abomasal secretion.

It does not follow that the abomasum is always capable of reducing the pH of its contents to low values. An important factor influencing gastric

emptying is the volume of the gastric contents (Hunt, 1959) since there may be situations where a high rate of flow from the forestomach could overwhelm the acid secretory capacity and result in a relatively high pH of the abomasal outflow. A low acidity of the material leaving the abomasum could also result from an inflow with a very high buffering or neutralizing capacity and a high rate of secretion with a low concentration of acid. The secretion of the pyloric antrum is unlikely to cause any large change in the pH of the contents leaving the abomasum. The rate of flow of secretion from pouches made from the whole of the antrum was 2–8 ml./hr with a bicarbonate concentration of 11–15 m-equiv/l. (Ash, 1959*b*). The total neutralizing and buffering capacity of this secretion is therefore very small when compared with the large volume of highly acid abomasal contents.

Clearly, it was the flow of food material *through* the abomasum of the intact animal which was responsible for the continuity and observed patterns of acid secretion. When the outflow of abomasal contents was prevented the secretion of acid increased temporarily and then declined to a low level. The initial increase was probably the result of increased abomasal distension, because flow into the abomasum from the forestomach may have continued until forestomach motility was reduced (Phillipson, 1939; Titchen, 1958, 1960; Stevens, Sellers & Spurrell, 1960), when the degree of distension reached a critical level. The large volumes of material which gushed from the abomasum when the pyloric cannula was opened support this idea. The decline in acid secretion was possibly the resultant of inhibition of inflow and an increase in the acidity of the abomasal contents to a value at which acid secretion was inhibited. The acidity of the material which gushed from the pyloric cannula was about pH 2·0; the introduction of solutions at pH 2·0 into the abomasum failed to stimulate the secretion of acid (Ash, 1959*a*). Additional evidence for the inhibitory effect of an increased acidity on acid secretion was provided by the experiments in which an artificial acid secretion was infused into the abomasum. It is necessary to attempt to explain how acid secretion was continuous when the acidity of the abomasal contents at the antrum was between pH 2–3 yet acid secretion declined at about pH 2. The inhibitory effect of acid may not depend entirely on the pH of the abomasal contents and values over 2 are probably above the inhibitory threshold under normal feeding conditions. Hoflund (1940) observed that the pH of the abomasal contents at the cranial end of the organ tended to be higher than that at the antrum, while the pH of the predigested food entering the abomasum from the forestomach is about 6 (P. K. Briggs, personal communication). A similar state of affairs has been shown to exist in the human stomach (James, 1957).

SUMMARY

1. A method of preparing sheep is described which allowed the simultaneous measurements of acid secretion by an abomasal pouch and the flow of food material from the abomasum.

2. When restricted feeding regimes were imposed on the animals, well defined patterns of acid secretion by the pouches were observed but there was no consistent response to feeding or to rumination.

3. There was a close correlation between acid secretion by the pouch and the outflow of food material from the abomasum. Increasing the food intake increased the amount of acid secreted, the volume of the abomasal outflow, the volume of water drunk and the time spent ruminating; decreasing the food intake caused the opposite effects.

4. At all rates of abomasal outflow the acidity of the material fluctuated only within narrow limits but large changes occurred in the output of acid by the pouch.

5. Preventing outflow did not inhibit acid secretion until the acidity of the abomasal contents increased to about pH 2·0.

6. When the flow of material from the abomasum was inhibited by distending the duodenum there was a simultaneous inhibition of acid secretion.

7. Infusions of artificial abomasal secretion (total HCl 80–115 m-equiv/l.) into the main body of the abomasum of normally fed sheep caused a profound inhibition of acid secretion from the pouches; the secretion obtained from one animal during inhibition contained bicarbonate. Isotonic NaCl solution did not inhibit acid secretion.

8. The flow of predigested food from the forestomach into the abomasum stimulated the secretion of acid but it was the rate of passage of this material through the abomasum which appeared to determine the pattern of acid secretion.

9. It is concluded that the inflow of food from the forestomach, acid secretion by the fundic glands and the flow of material from the abomasum are normally integrated and that the acidity of the abomasal contents is of major importance in the control of acid secretion.

I wish to thank Dr A. T. Phillipson for many helpful discussions throughout the course of the work. Mr J. Dunnett and Miss Mary Smith are thanked for their skilled technical assistance and enthusiastic care of the experimental animals.

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THE MECHANICAL PROPERTIES OF THE UTERINE CERVIX OF THE RAT DURING INVOLUTION AFTER PARTURITION

By MARGARET L. R. HARKNESS and R. D. HARKNESS
From the Department of Physiology, University College London

(*Received* 10 *October* 1960)

We have previously described the change in mechanical properties of the uterine cervix of the rat which enables the foetus to pass through this normally narrow rigid canal at the end of pregnancy (Harkness & Harkness, 1959*b*). It was found that there was an increase in the circumference of the collagenous framework of the cervix, together with a change in its nature, so that it became readily distensible by prolonged small forces. We have now studied the reversal of these changes after parturition and their relation to the rapid removal of collagen from the uterus and cervix which takes place at this time (Harkness & Harkness, 1954; Harkness & Moralee, 1956). Within 24 hr after parturition, the circumference of the collagenous framework decreases to one third of the value at parturition. The property of extensibility under prolonged loading is lost. There is, subsequently, a slower return of the circumference to approximately the non-pregnant value. The alterations in the mechanical properties of the cervix take place more rapidly than loss of collagen. A preliminary account of this work has already been published (Harkness & Harkness, 1958).

METHODS

The animals were albino rats of the local strain. The methods used for examining the excised cervix have previously been described (Harkness & Harkness, 1959*b*; Cullen & Harkness, 1960). It is sufficient to say that two types of measurement were made.

Dimensional. The inner circumference of the cervix at all levels of tension, applied rapidly (minutes) to the point of rupture (from which the *tensile strength* was derived). Symbols: l_a, circumference (mm) at zero tension obtained by linear extrapolation (= *a*, Harkness & Harkness, 1959*b*); k, the difference between l_a and circumference at breaking tension (= *b*, Harkness & Harkness, 1959*b*); l_1 circumference (mm) under one standard condition (see Harkness & Harkness, 1959*b*).

Viscous extensibility. The rate of increase in circumference under prolonged tension (hours); the term 'viscous' extensibility is used, since the tissues under test behave like a viscous element in a rheological model, extending at constant rate under a constant load. Symbols: K/l_0 where K is rate of increase in circumference (mm/min) and l_0 is circumference (mm) obtained by linear extrapolation to zero time. K/l_0 is, therefore, the fractional increase in circumference per unit time. The relation of this to tension is not a simple proportionality and has not yet been fully worked out. Comparisons between tissues may be made at known values of tension.

TABLE 1. Composition and mechanical properties of cervix at different times after parturition

	During parturition			2–4 hr	4–8 hr			1 day		2 days	3 days	4 days	8 days	16 days
	†22°	37°	22° (Pap)	22°	22°	37°	22° (Pap)	22°	37°	22°	22°	22°	22°	22°
1. No. of rats	4	5	4	4	5	4	4	4	2	6	5	6	5	5
2. Body weight at death (g)	217 ±19	239 ±9	268 ±18	199 ±24	199 ±8	192 ±16	205 ±15	191 ±9	188 ±18	198 ±7	188 ±9	226 ±5	224 ±11	255 ±18
3. Weight of cervix (mg)	258 ±28	252 ±26	273 ±12	255 ±4	204 ±10	209 ±34	252 ±14	146 ±16	131 ±3	107 ±5	75 ±7	55 ±2	35 ±2	29 ±2
4. Total collagen in cervix (mg)	0·9 ±1·0	0·5 ±0·7	0·8 ±0·3	0·6 ±0·8	0·9 ±0·7	0·5 ±0·8	0·7 ±0·2	0·7 ±0·4	0·6 ±0·6	0·4 ±0·4	0·8 ±0·6	0·2 ±0·4	0·1 ±0·1	0·0 ±0·2
5. Concn. of collagen (g/100 g)	3·9 ±0·1	3·8 ±0·2	3·6 ±0·2	3·8 ±0·3	4·3 ±0·2	4·4 ±0·7	3·9 ±0·2	6·1 ±0·3	6·6 ±0·6	7·0 ±0·2	6·5 ±0·2	7·5 ±0·7	6·7 ±0·2	9·0 ±0·5
6. l_1 (mm)	35·5 ±1·6	46·7 ±4·4	35·4 ±0·6	24·7 ±1·1	9·3 ±1·1	10·2 ±0·8	12·5 ±1·4	9·1 ±0·3	10·9 ±0·1	8·9 ±0·3	7·5 ±0·2	6·5 ±0·3	4·7 ±0·1	4·1 ±0·1
7. l_2 (mm)	43·7 ±2·3	*67·8 ±9·8	50·0 ±3·2	35·9 ±0·7	12·2 ±2·2	8·9 ±2·2	14·9 ±2·9	11·7 ±0·6	12·2 ±0·3	11·6 ±0·7	9·7 ±0·4	8·2 ±0·3	5·2 ±0·1	4·7 ±0·1
8. k/l_2	0·29 ±0·03	*0·20 ±0·03	0·22 ±0·02	0·37 ±0·05	0·48 ±0·04	0·51 ±0·17	0·51 ±0·04	0·37 ±0·02	0·44 ±0·01	0·41 ±0·04	0·38 ±0·02	0·37 ±0·03	0·33 ±0·05	0·30 ±0·06
9. Break circumference (mm)	56·8 ±2·9	79·3 ±7·0	61·3 ±4·4	51·2 ±2·9	19·4 ±3·2	19·4 ±2·8	23·3 ±4·1	16·2 ±0·6	17·8 ±0·4	16·5 ±0·9	13·5 ±0·4	11·4 ±0·6	7·5 ±0·5	6·2 ±0·4
10. Break tension (kg/mm² collagen)	1·13 ±0·12	*0·68 ±0·14	1·19 ±0·10	1·28 ±0·09	0·87 ±0·17	0·74 ±0·13	1·01 ±0·21	1·41 ±0·10	0·86 ±0·10	0·99 ±0·09	1·13 ±0·08	1·09 ±0·14	0·84 ±0·11	0·84 ±0·09

The estimate of variation is the standard error of the mean. The meanings of l_1, l_2 and k are given in the text.

† Temperature of test.

* Based on 3 animals in which cervix did not rupture under initial 50 g loading. (Pap) = tests done in presence of papaverine sulphate 50 mg/l.

Chemical

All samples were placed in 5 ml. 6N-HCl at the end of tests and hydrolysed for 4 hr at 40 lb./sq.in. (2·8 kg/cm²) pressure in an autoclave. The hydroxyproline content of the hydrolysate was estimated by the method of Neuman & Logan (1950), and the collagen content of the sample estimated by multiplying this by 7·46.

RESULTS

Circumference of cervix. The results of experiments concerned with the changes in dimensions of the cervix are recorded in Table 1 and Figs. 1 and 2. Circumference (line 7, Table 1) falls in 8 hr to about one third of that at parturition. Subsequently there is a further, slower fall, until by the eighth day the value is about an eighth of that at parturition. There is also a fall in the weight of cervix (line 3), accompanied by loss of collagen (line 4), these changes becoming evident 24 hr after parturition. The latter change proceeds more slowly so that there is a rise in concentration of collagen.

Tensile strength of tissue. The absolute load required to break the tissues rose after parturition and then fell (Fig. 3). The explanation appears to be that at first the walls of the cervix become thicker, because the fall in circumference proceeds faster than loss of tissue, and the breaking load rises. Later there is a loss of tissue, and the walls become thinner and hence weaker. Breaking tension per unit cross-sectional area of collagen shows comparatively little variation over the whole period of study (Table 1, line 10). The figures in Table 1 are calculated from inner circumference. If mean circumference is estimated (mean between inner and outer) and used instead, the correction at the early stages after parturition is small (about 5%) but rises to about 30% at 16 days. The effect is to reduce the differences in tensile strength per unit cross-sectional area of collagen between groups.

Extensibility. Measurements were made at only one time (24 hr) after parturition. Results are given in Table 2. Values of K/l_0 for the comparable tension per unit cross-sectional area of collagen 24 hr after parturition are similar to those found in the normal cervix, and 30–40 times lower than at parturition (Harkness & Harkness, 1959*b*).

TABLE 2. Extensibility of cervix 24 hr after parturition

l_i (mm)	16·8 ± 1·7
l_0 (mm)	18·1 ± 1·8
$10^3\ K/l_0$	0·59 ± 0·18
Tension at l_0 (g/mm² collagen)	490 ± 77
Tension at break (kg/mm² collagen)	1·55 ± 0·19

The estimate of variation is the standard error of the mean.

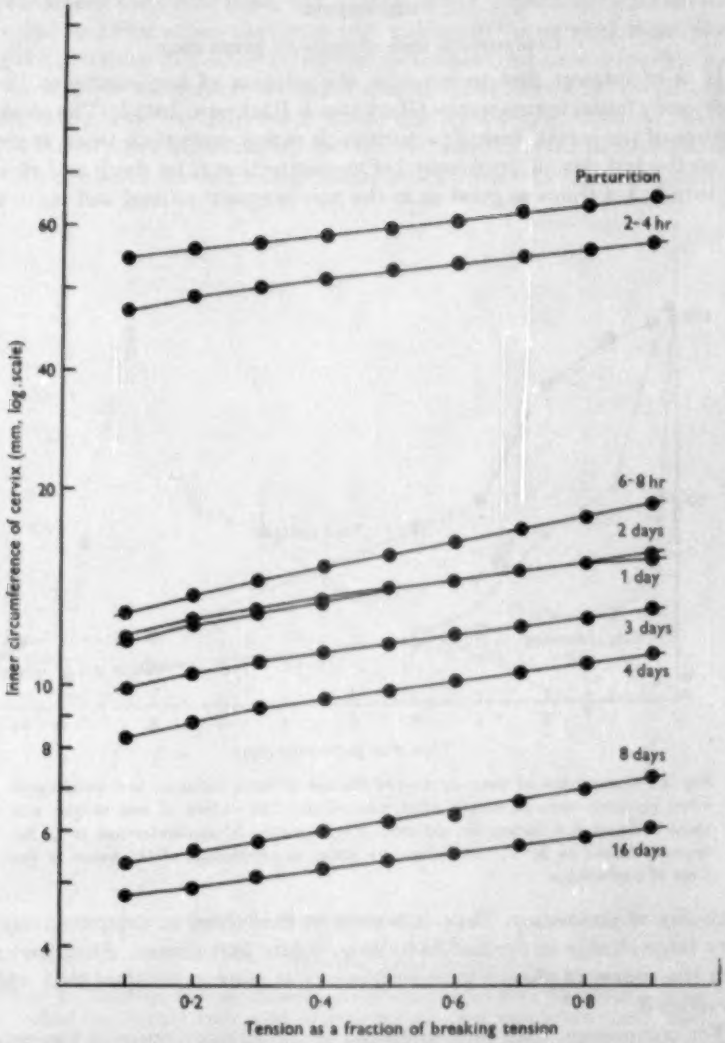

Fig. 1. The relation of inner circumference of cervix to tension. The curves represent mean values for all animals in each group. The time after parturition is given against each curve. Tension (abscissa) is given as a fraction of breaking tension. Samples tested at 22° C. Semi-log. scale.

DISCUSSION

Comparison with changes in pregnancy

It is of interest first to consider the relation of our results to those previously found in pregnancy (Harkness & Harkness, 1959*b*). The circumference of the cervix during parturition is rather more than twice as great as on the last day of pregnancy before parturition (21st day), and this in its turn is 3–4 times as great as in the non-pregnant animal and up to the

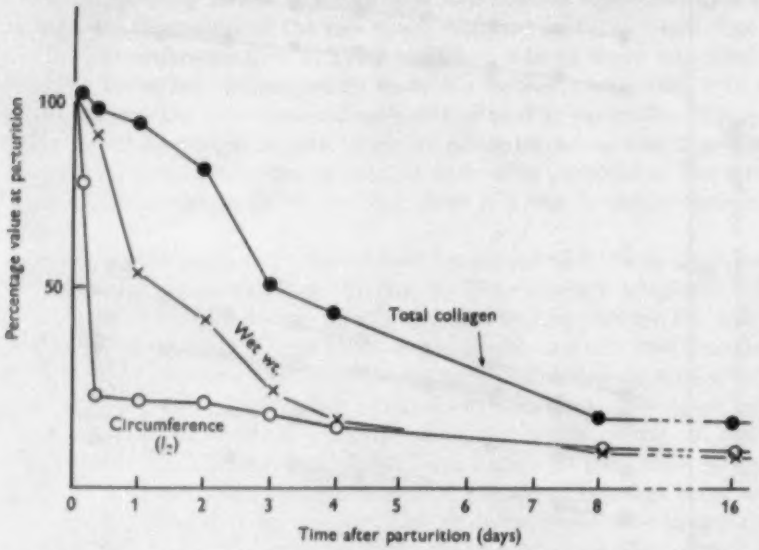

Fig. 2. Comparison of time courses of change in total collagen, wet weight and inner circumference of cervix after parturition. The values of wet weight and total collagen are means for all rats. The measure of circumference is l_2, for samples tested at 22° C. All values are given as percentage of the value at the time of parturition.

12th day of pregnancy. Thus, it is evident that there is, comparatively, a very large change in the last 24 hr or so before parturition. After parturition the course of change in circumference is more rapid than that which precedes it.

For comparison with measurements of the circumference of the cervix made during parturition it may be stated that the circumference of the foetal head is about 35 mm (Harkness & Harkness, 1959*b*). This is rather less than the value of l_2, obtained at parturition by extrapolation of the length–tension curve to zero load, which may be taken as a measure of the size of the unstretched collagenous framework. The reason l_2 is not equal

to the size of the foetal head, but slightly larger, is probably because there is a thin partition across the upper part at least of the cervical canal, dividing the two sides so that an individual foetus does not have unhindered use of the whole circumference we have measured, but dilates the cervix asymmetrically. This explanation is borne out by a single instance that we observed of a rat, killed during parturition, which proved to have foetuses in only one horn. In this animal dilatation of the cervix was asymmetrical.

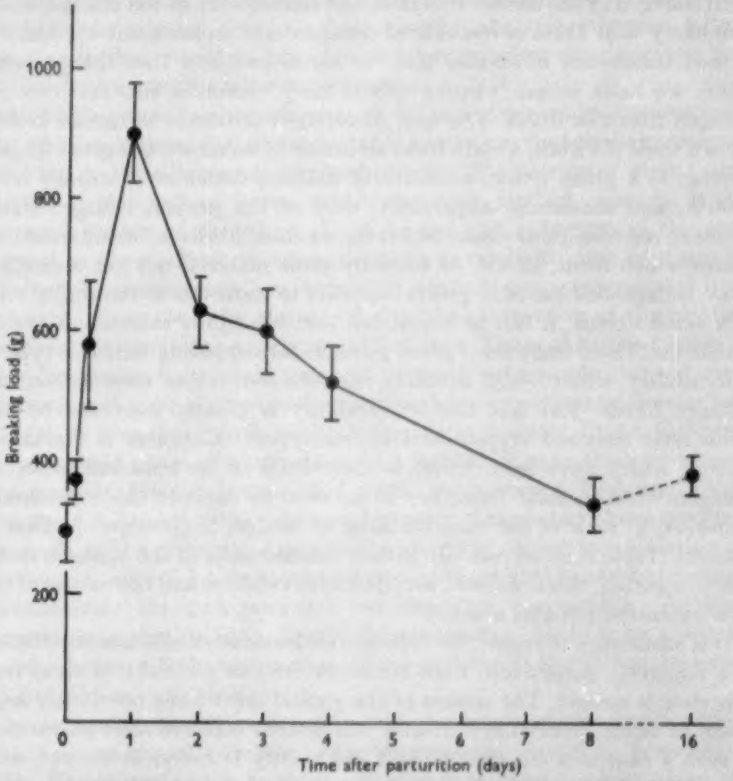

Fig. 3. Breaking load at different times after parturition. The length of the vertical line through each point is twice the standard error of the mean. Tests done at 22° C.

Changes in the first day after parturition

The changes after parturition fall roughly into two phases, an early one of rapid change and consolidation of the tissue in the first day, and a later one of slow change associated with the general involution of the reproductive tract which occurs *post partum* after the first 24 hr. In the first

phase, immediately after parturition, a reduction in circumference is presumably brought about by contraction of smooth muscle. This, however, is rapidly made permanent by a consolidation of the tissue; the property which we have called viscous extensibility is lost, and the tissues cannot be stretched again. It is obvious from the nature of this consolidation that it cannot be the result of muscular activity, but must reflect a change in the collagenous framework; it takes place with little or no alteration in the total collagen of the tissue. This fact, and the rapidity of the change, make it unlikely that there is removal of collagen and replacement by a newly formed framework of smaller size. It seems probable that the property which we have called 'viscous extensibility' involves slip between the collagen fibrils or fibres. The unit of collagen structure in tissues is well known to be the fibril, a cylindrical structure of unknown but great length, having, in a given tissue, a relatively uniform diameter of usually 500–1000 Å, and consisting, apparently, only of the protein collagen itself. Electron microscopical examination shows these fibrils to be separated by spaces which must, in life, be filled by some material not yet identified. Since collagenous tissue in general appears to show this extensibility only to a small extent, it can be concluded that the fibrils themselves are inextensible. Thus, there are *a priori* grounds for supposing that this type of extensibility, when found, involves slip between, rather than within, the collagen fibrils. The fact that extensibility is greatly increased by the proteolytic enzymes trypsin and chymotrypsin (Harkness & Harkness, 1959*a*), which have been found to have little or no apparent effect on collagen when in solid form, has to be used to support this hypothesis. However, in view of the recent finding of Hodge, Highberger, Deffner & Schmitt (1960) that trypsin can attack the free ends of the collagen molecule in solution, this argument is of doubtful validity, and the nature of the slow extension remains obscure.

It is customary to regard the collagenous framework of connective tissue as a relatively permanent, fixed structure, and in general it is clear that this view is correct. The tissues of the genital tract have previously been found to be exceptional in that they can destroy collagen after parturition at such a rate that the time to half loss is only 1–2 days in the rat, considerably less than the half life, in this animal, of such metabolically active material as plasma protein. It is now clear that still more rapid changes can take place in the mechanical properties of the collagenous framework of the genital tract, but it seems unlikely that the physiological importance of the properties we have been investigating is confined to this tract. One might expect, for example, that similar changes are involved in the closure of the ductus arteriosus after birth.

Comparison of later changes with contraction in wounds

In the second phase of the changes in the cervix, from 24 hr on, there is a much slower fall in circumference which roughly parallels the general involution of the tissue, shown by fall in weight and total collagen content. Change in circumference, which is a linear dimension, cannot be compared directly with change in total collagen content, but might be expected, if the two were linked, to be proportional to the cube root of the latter. Although the relation does not hold exactly, the circumference falling rather more than would be expected, it is nevertheless clear that the two changes proceed with similar time courses.

The later change in circumference of the cervix resembles the 'contraction' of wound tissue, and it is reasonable to suppose that similar processes in the connective tissues are involved in both. The process of wound contraction in skin, where it has principally been studied, consists in the drawing together of the edges of the wound and reduction in the area enclosed by them. Both processes diminish in rate with time, and can be described, approximately, in terms of one or more exponential time constants. From 24 hr to the eighth day the contraction of the circumference of the cervix has a half time of 5–6 days. Times of half reduction of linear dimensions for wounds, assumed proportional to square root of area, vary both with time after wounding and between experiments, from as low as 2–4 days (Van den Brenk, 1956; Grillo, Watts & Gross, 1958*a*, *b*) during the most rapid phase of contraction, to 2 months or so in the later phases (Abercrombie, Flint & James, 1954, 1956; Billingham & Medawar, 1955; Billingham & Russell, 1956). Although greater changes have been reported in wounds, in the majority of reports the extent of contraction is similar to that found in cervix, i.e. to between $\frac{1}{2}$ and $\frac{1}{3}$ the original linear dimensions. Quantitatively the two processes are therefore comparable. Another resemblance is that in both, during the contraction, there is an absolute loss of substance from contracting tissue and the material lost includes collagen (Grillo *et al.* 1958*a*, *b*).

The nature of wound contraction and the origin of the forces which bring it about are still disputed (see Abercrombie, James & Newcombe, 1960). Experiments such as those reported here have not been done on wounds, but it is reasonable to suppose that in them also the contraction involves a diminution in the size of the collagenous framework. None of the hypotheses so far put forward has taken this into consideration.

SUMMARY

1. Changes in the circumference and mechanical properties of the cervix uteri of the rat, from the time of parturition to 16 days after, have

been investigated by stretching the tissue between two parallel rods, one through each canal.

2. There was a rapid decrease within 8 hr in the circumference of the collagenous framework of the cervix, to about one third the value at parturition, followed by a slower reduction to a third again in the next 2 weeks to a value near the normal.

3. The initial decrease in circumference takes place with little change in the total collagen content of the tissue, but the later change is associated with the general involution of the tissue which occurs after parturition.

4. Within 24 hr after parturition the collagenous framework of the cervix loses the ability which it develops during pregnancy, to extend continuously under prolonged low loads, and its behaviour resembles that of a normal non-pregnant cervix.

We are very grateful to Miss Shirley M. Fitch for her skilled technical assistance.

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SUPERSENSITIVITY OF SALIVARY GLAND CAUSED BY BOTULINUM TOXIN

BY N. EMMELIN

From the Institute of Physiology, University of Lund, Sweden

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Certain pharmacological agents interfering with the transmission of nerve impulses have been found to sensitize effector cells to chemical stimuli if allowed to act for some days. Prolonged treatment with parasympatholytic substances increases the responsiveness of salivary and lachrymal glands and of the smooth muscle of the stomach (Emmelin & Muren, 1950, 1952; Emmelin & Strömblad, 1956, 1957; Strömblad, 1956; Muren, 1957). Ganglion-blocking compounds, administered for some time, sensitize the submaxillary gland and the nictitating membrane (Emmelin, 1959). Treatment with reserpine causes a supersensitivity in various organs supplied with adrenergic nerves (Burn & Rand, 1959). Recently Thesleff (1960) has shown that botulinum toxin, injected into the musculature of the cat's hind leg or applied to the exposed surface of the tenuissimus muscle, produces a supersensitivity in this muscle which is similar to that seen after severance of the motor nerve (Axelsson & Thesleff, 1959).

In the present experiments the effect of botulinum toxin on the sensitivity of the cat's submaxillary and parotid glands was investigated.

METHODS

Twenty cats were used for these experiments. About once a week the level of sensitivity of the salivary glands was estimated, with a short-acting barbiturate as an anaesthetic, as earlier described (Emmelin & Muren, 1952; Emmelin & Engström, 1960). Hexobarbitone was given when adrenaline and dopamine were used to evoke secretion, and Baytinal Bayer (sodium 5,5-allyl-(2′-methyl-propyl)-thiobarbituricum) when methacholine and acetylcholine were used for this purpose. In the latter case hexamethonium 5 mg/kg was administered intracardially to abolish any reflex secretion. This is not required in hexobarbitone anaesthesia owing to the atropine-like effect of the anaesthetic.

To estimate the sensitivity standard doses were injected of adrenaline (0·5, 1, 2, 5 and 10 μg/kg), of dopamine (0·2 and 0·5 mg/kg), of methacholine (0·25, 0·5, 1 and 2 μg/kg), and of acetylcholine (0·5, 1 and 2 μg/kg). For submaxillary glands adrenaline and dopamine were used as a routine; occasionally methacholine and acetylcholine were given. Parotid glands, very insensitive to sympathomimetic drugs, were stimulated with methacholine only.

When the level of sensitivity of the normal gland had been determined, botulinum toxin, type A, was injected into the gland from a syringe, attached by a polythene tube to a fine glass cannula temporarily inserted from the mouth into the salivary duct. The amount of toxin given varied between 0·2 and 50 μg, dissolved in 0·1–0·2 ml. saline solution. Before

this injection 1 mg/kg of a parasympatholytic drug (αα-diphenyl-γ-piperidinobutyramide, Hoechst 9980) was given intracardially in most cases. In some experiments 0·2 ml. saline solution was injected through the contralateral duct as a control.

RESULTS

In the first experiments doses of the toxin of 10–50 μg were used. In some of these cases the sensitivity of the gland injected was found to start to rise, but the animals died within a week. Doses of 0·2–1 μg were usually tolerated but did not cause any marked sensitization. In order to abolish, for some hours, all reflex secretion, which could wash out the toxin, the drug Hoechst 9980 was introduced; the dose of the toxin given was then always 1 μg. This procedure was regularly found to cause a marked supersensitivity. The single dose of the parasympatholytic alone did not cause any sensitization, nor did the control injection of saline solution into the duct.

Submaxillary glands. Figure 1 shows the results of an experiment on the submaxillary gland, carried out over a period of 4 weeks. The secretory

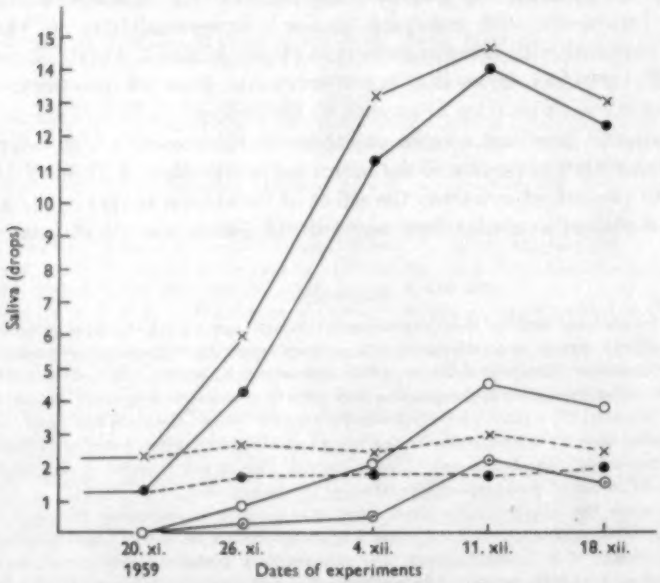

Fig. 1. Effect of botulinum toxin on the submaxillary gland. Ordinates: drops of saliva in response to different doses of adrenaline. Abscissa: dates of experiments. On 20 November 1 μg of the toxin was given into the right duct. Solid lines correspond to right, broken lines to left gland. – – – gland. Responses to adrenaline: × 10 μg/kg, ● 5, ○ 2, ⊙ 1 μg/kg.

responses to four different doses of adrenaline can be seen. One week after the injection of botulinum toxin a supersensitivity was obvious and it increased and had reached a maximum after 3 weeks. A similar picture is seen when the chorda tympani is cut or treatment with a parasympatholytic agent given. In all three cases the threshold dose is lowered, and the responses to moderate doses of the secretory agent raised.

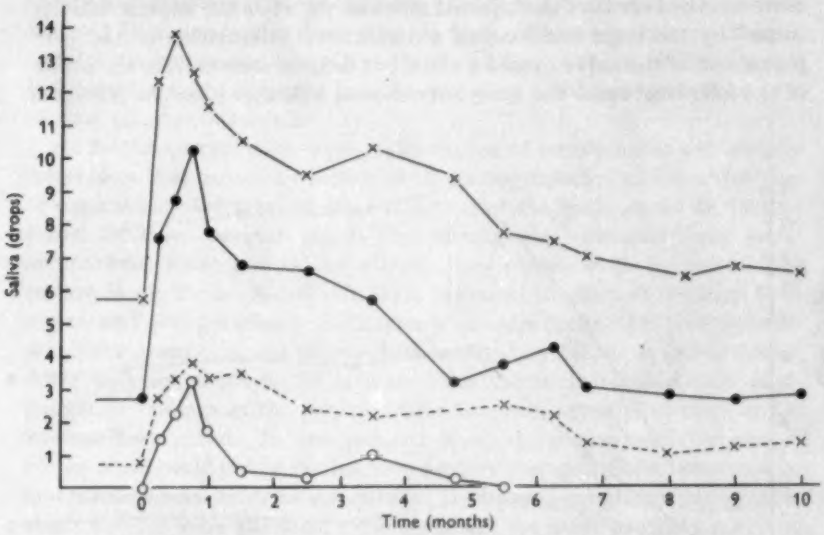

Fig. 2. Submaxillary gland. Supersensitivity caused by 1 μg botulinum toxin injected at zero time. Solid lines: responses of injected gland to adrenaline: × 10, ● 5, ○ 2 μg/kg. Interrupted line: responses to dopamine 0·5 mg/kg.

After 3–4 weeks a small decline in the responses to the standard doses could be seen. This is found after severance of the chorda also. In both instances it is probably due to atrophy of the gland. In one cat the glands were extirpated 1 month after the injection of botulinum toxin. The injected gland weighed 0·650 g, the contralateral gland 1·078 g. In another cat the corresponding figures 3 weeks after injection of the toxin were 0·980 and 1·260 g, respectively.

When the sensitivity was observed over a long period of time it was found to continue to fall off, as shown in Fig. 2. Eventually, after about 8 months, the pre-treatment level had been reached. The animal was now killed in an acute experiment. Chorda stimulation was found to be as effective on the injected as on the untreated gland; the former gland weighed 1·225 g, the latter one 1·265 g. Obviously the effect of the toxin had slowly worn off and normal conditions had been restored.

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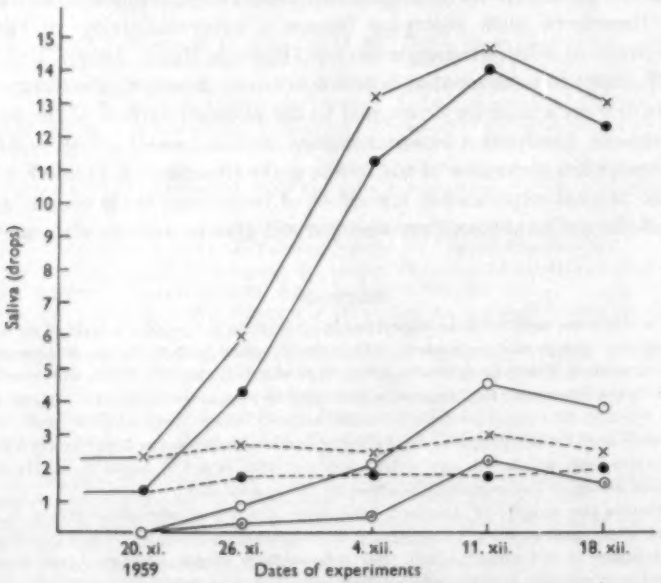

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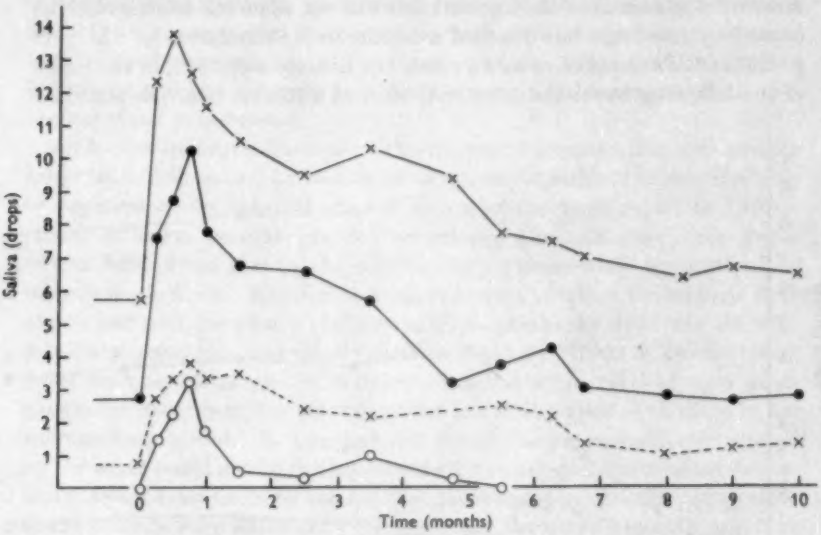

Fig. 2. Submaxillary gland. Supersensitivity caused by 1 μg botulinum toxin injected at zero time. Solid lines: responses of injected gland to adrenaline: × 10, ● 5, ○ 2 μg/kg. Interrupted line: responses to dopamine 0·5 mg/kg.

After 3–4 weeks a small decline in the responses to the standard doses could be seen. This is found after severance of the chorda also. In both instances it is probably due to atrophy of the gland. In one cat the glands were extirpated 1 month after the injection of botulinum toxin. The injected gland weighed 0·650 g, the contralateral gland 1·078 g. In another cat the corresponding figures 3 weeks after injection of the toxin were 0·980 and 1·260 g, respectively.

When the sensitivity was observed over a long period of time it was found to continue to fall off, as shown in Fig. 2. Eventually, after about 8 months, the pre-treatment level had been reached. The animal was now killed in an acute experiment. Chorda stimulation was found to be as effective on the injected as on the untreated gland; the former gland weighed 1·225 g, the latter one 1·265 g. Obviously the effect of the toxin had slowly worn off and normal conditions had been restored.

Supersensitivity towards dopamine, acetylcholine and methacholine was also found to develop in submaxillary glands treated with botulinum toxin.

The level of supersensitivity reached after injection of the toxin was high, as can be seen in Figs. 1 and 2. Whether it was as high as that attainable by section of the chorda, or higher, in these two cases is not known, since the chorda was not cut in these cats. In three other experiments, however, the chorda of the injected side was cut when the supersensitivity caused by the toxin had reached a maximum. Stimulation of the peripheral end of the nerve caused a small but definite secretion; in the course of the following weeks the sensitivity showed a further rise. The chorda of

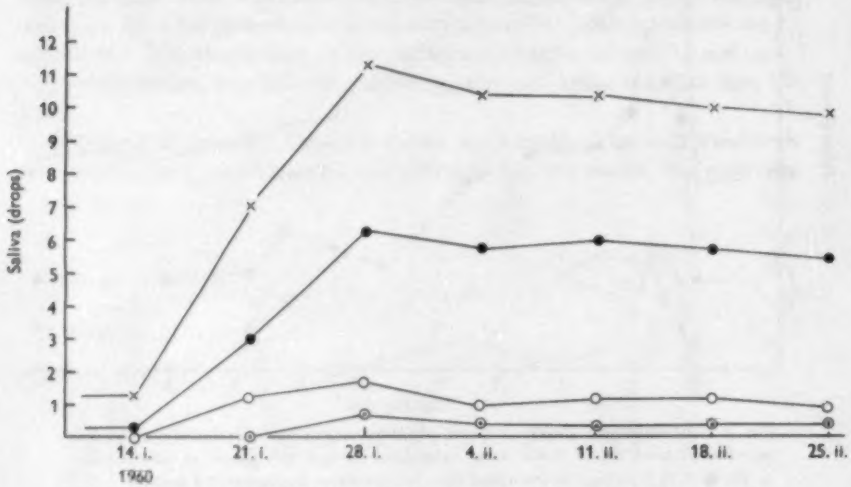

Fig. 3. Supersensitivity of a parotid gland caused by 1 μg botulinum toxin injected through the duct on 14 January. Drops of saliva (ordinates) in response to methacholine; × 2 μg/kg, ● 1, ○ 0·5 and ⊙ 0·25 μg/kg. On 11 February the auriculo-temporal nerve fibres were cut.

the contralateral submaxillary gland was then cut. The supersensitivity that ensued was found to be slightly lower than that of the gland subjected both to botulinum toxin and section of the chorda. In one of these cats treatment with the parasympatholytic drug Hoechst 9980 was later given. There was a further increase in sensitivity on both sides; and the two glands reached the same level of supersensitivity.

Parotid glands. Botulinum toxin injected through the parotid duct caused a supersensitivity in the parotid gland. The sensitizing effect towards four different doses of methacholine is shown in Fig. 3. The parotid gland was treated with botulinum toxin in five cats. When a fairly constant degree of supersensitivity had developed, the post-ganglionic para-

sympathetic fibres of the glands were cut. In four of the glands this resulted in a further rise in the sensitivity. In one gland, the one shown in Fig. 3, this was not the case.

DISCUSSION

The supersensitivity following injection of botulinum toxin into the salivary glands resembles that caused by parasympathetic denervation in the following respects. It develops gradually and shows the same time course. It is unspecific, demonstrable both with sympathomimetic and parasympathomimetic drugs. The threshold dose of the stimulating agent is lowered and the response to a dose causing a submaximal effect on a normal gland is increased.

As to the quantitative aspect, the degree of sensitization was usually lower than that caused by section of the parasympathetic fibres. The level of supersensitivity reached after injection of the toxin could be further raised in most parotid glands by cutting the post-ganglionic parasympathetic fibres and in the submaxillary glands even by cutting the preganglionic fibres. Botulinum toxin is known to affect the endings both of pre- and post-ganglionic cholinergic fibres (Ambache, 1951); in the submaxillary gland this was clearly demonstrated by Hilton & Lewis (1955). After injection through the salivary duct the toxin reached only post-ganglionic endings in the parotid gland but both types of endings in the submaxillary gland. In one parotid gland the supersensitivity caused by the toxin could not be further increased by post-ganglionic denervation, and it seems reasonable to assume that all the post-ganglionic parasympathetic endings were affected by the toxin. In the other parotid glands the blockade by the toxin was probably incomplete. In the submaxillary glands the effect of the toxin was found to be incomplete: stimulation of the chorda produced secretion, showing that some pre- and post-ganglionic endings could still function. Section of the chorda increased the supersensitivity; it increased it above the level reached after cutting this nerve in a non-intoxicated gland. This can be interpreted to mean that the toxin had produced a partial post-ganglionic 'denervation'; for it is known that cutting the chorda and dissecting it as close to the hilum as possible, thus causing a complete preganglionic and a partial post-ganglionic parasympathetic denervation, evokes a supersensitivity more pronounced than that caused by cutting the chorda alone (Emmelin, 1960*a*). Not only post-ganglionic but preganglionic endings also were probably affected by the toxin in these experiments; Hilton & Lewis (1955) found the effect of preganglionic stimulation easier to abolish with botulinum toxin than that of post-ganglionic stimulation.

The finding that the supersensitivity caused by botulinum toxin cannot be superimposed upon that produced by prolonged 'atropinization' is in

keeping with the interpretation given above, since the latter procedure corresponds to a complete post-ganglionic parasympathetic denervation. The fact that such a complete 'denervation' was attained only in one case with botulinum toxin is no doubt due to insufficient dosage; but the dose used seemed to be the highest one that could safely be used in long-term experiments.

Botulinum toxin is assumed to interfere with the release of acetylcholine (Burgen, Dickens & Zatman, 1949; Thesleff, 1960). The fact that it causes a supersensitivity seems to afford additional evidence to show that acetylcholine normally exerts some action to keep the sensitivity of the salivary gland cells low (see Emmelin, 1960*b*).

In most of the experiments a small dose of the parasympatholytic drug Hoechst 9980 was given intracardially before the injection of the botulinum toxin. This drug is able to sensitize the gland cells, but to do so it has to be given repeatedly. It may be pointed out that the gland not receiving the toxin injection showed no supersensitivity; furthermore, in the early experiments the toxin was given alone and the development of a supersensitivity could clearly be detected before the animals died from botulinum intoxication. These observations support the view that the supersensitivity studied in this investigation was due to the toxin; the very long-lasting effect points in the same direction. The function of the parasympatholytic was merely to suppress salivary secretion for some time after the administration of the toxin. Fixation of the toxin to the gland was thereby favoured, the local sensitizing effect was promoted and the risk of general intoxication from swallowed toxin diminished.

SUMMARY

1. Cats were anaesthetized repeatedly with a short-acting barbiturate, the submaxillary or parotid ducts cannulated from the mouth and the secretory responses to standard doses of adrenaline, dopamine, acetylcholine and methacholine estimated.

2. Injection of botulinum toxin, type A, through the salivary duct towards the gland was found to produce a supersensitivity towards the secretory agents. The sensitivity rose gradually, reaching a maximum within about 3 weeks and declining slowly in the course of about 8 months to the pre-treatment level.

3. The supersensitivity resembled that caused by parasympathetic denervation of the glands. It is inferred that the sensitization in both cases is due to lack of some action of acetylcholine on the gland cells.

Dr S. Thesleff of the Department of Pharmacology, University of Lund, very kindly supplied the botulinum toxin and helped with its injection.

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EFFECT OF EXERCISE ON THE LEVEL OF ANTIHAEMOPHILIC GLOBULIN IN HUMAN BLOOD

By C. R. RIZZA*

From the Blood Coagulation Research Unit, Churchill Hospital, Oxford

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It is thought that the coagulation defect in haemophilia is due to the absence of an essential blood-clotting factor. This factor, called antihaemophilic globulin (AHG), is present in fresh normal blood, but deteriorates when blood is stored. At present the only way of correcting the haemostatic and coagulation defect in haemophilia is by transfusions of blood, plasma or plasma protein concentrates rich in antihaemophilic globulin, and it is therefore desirable to understand the mechanism whereby AHG activity disappears from stored blood and plasma.

The fibrinolytic enzyme, plasmin, is one of the agents which is known to destroy AHG (Wagner, Pate & Brinkhous, 1954; Brakman, van Creveld, Engelsman, van't Laar, Mochtar & Veder, 1959) and Penick & Brinkhous (1956) have suggested that the loss of AHG activity of blood on storage might be due to the presence of traces of plasmin.

Biggs, Macfarlane & Pilling (1947) demonstrated increased fibrinolytic activity in the blood of normal people after vigorous exercise. This observation seemed to provide a satisfactory way of studying the effect of the naturally occurring fibrinolytic enzyme on the stability of AHG in stored blood, and experiments were done in which the deterioration of AHG was studied in the blood collected from healthy subjects before and after exercise. In the course of the investigation it was observed that the blood samples taken after exercise showed much more AHG activity than the samples taken before exercise. This unexpected finding is the subject of this paper.

METHODS

Blood was collected by venepuncture into a silicone-coated syringe and mixed with 1/9 volume of trisodium citrate (3·8 g/100 ml.) in a silicone-coated tube. The blood was then spun at 700 *g* for 10 min and the supernatant plasma separated.

Assay of AHG was performed according to the method of Biggs, Eveling & Richards (1955). The AHG level of the blood after exercise was expressed as a percentage of the level before exercise. In the experiments in which plasma was infused into haemophiliacs the AHG levels were expressed as a percentage of a standard pool of four normal plasma samples.

* MRC Clinical Research Fellow.

Factor-V-deficient plasma was prepared by incubating oxalated plasma at 37° C for 36 hr.

Factor V was assayed by determining the ability of the test plasma to correct the prolonged one-stage prothrombin time of Factor-V-deficient plasma (Owren, 1947).

Fibrinogen concentration of plasma was estimated by clotting the diluted plasma with 0·025 M-$CaCl_2$ and then carrying out a standard micro-Kjeldahl digestion on the washed clot.

Prothrombin was measured by the two-stage method of Biggs & Douglas (1953*a*).

Christmas factor was assayed according to the method of Biggs, Bidwell, Handley, Macfarlane, Trueta, Elliot-Smith, Dike & Ash (1961). This assay is based on the thromboplastin-generation test of Biggs & Douglas (1953*b*) and measures the ability of a test serum to correct the defective thromboplastin generation in the serum of a patient suffering from Christmas disease.

The one-stage prothrombin time was determined by a modification of Quick's one-stage procedure as described by Biggs & Macfarlane (1957).

The whole-blood clotting time and *the plasma recalcification time* in glass and silicone-coated tubes were carried out as described by Biggs & Macfarlane (1957).

The thromboplastin-generation test was carried out as described by Biggs & Douglas (1953*b*), except that a chloroform extract of brain was used in place of a platelet suspension.

The prothrombin consumption test was performed according to the method of Merskey (1950) and expressed as an index.

Fibrinolytic activity in citrated plasma was demonstrated by the method of Macfarlane & Biggs (1946).

Exercise procedure. All the subjects performed the same exercise, which entailed running as fast as possible around the hospital perimeter, a distance of approximately ¾ mile (1·2 km). The time taken to complete the run was 5–8 min and varied with the physical fitness and age of the subject. Blood samples were taken by venepuncture immediately before and immediately after exercise and in some cases at varying intervals throughout the day.

RESULTS

Effect of exercise on AHG activity in the blood of normal subjects

The effect of exercise on the level of AHG activity in the blood of normal people was studied in 18 experiments on fifteen subjects (eleven males, four females, aged 18–55 years). In each case there was a marked increase in the AHG activity of the blood after exercise, irrespective of the age or sex of the subject (Table 1).

Additional blood samples were obtained from four of the above subjects at intervals of 2, 4 and 6 hr after exercise, in order to see how long the level of AHG activity would remain raised. The average AHG level of blood taken from four subjects 6 hr after exercise was still about 60% higher than the control level. There was considerable variation from person to person in the percentage increase of AHG after exercise and the response of the same individual varied from day to day, as is shown by the figures for subjects 2 and 5 in Table 1.

In the above experiments the exercise performed was strenuous. The effect of less vigorous exercise on the AHG level was studied in five normal subjects after they had walked 3 miles (4·8 Km) and 6 miles at an average speed of 5 miles/hr. This amount of exercise failed to raise the AHG level.

After completing the 6 mile walk, two of the subjects ran ¾ mile as fast as possible. The level of AHG in both cases rose to 200% of the level before exercise.

Effect of repeated venepuncture on AHG activity in the blood of normal subjects

To investigate the possibility that the trauma and pain of venepuncture might in some way raise the level of AHG activity, blood samples were taken at 10 min intervals from six healthy volunteers (three males, three females) at rest. Four of the subjects had three venepunctures and the remainder had two each. There was no consistent change in the AHG level with successive venepunctures, the average levels being 101% after the second venepuncture and 97% after the third venepuncture compared with an initial level of 100%. All the subjects were accustomed to venepuncture and in previous experiments all had shown a rise of AHG after exercise.

TABLE 1. Effect of exercise on plasma AHG level of normal subjects

Subject	Age	Sex	Level of AHG after exercise (% of level before exercise)
1	28	*M*	200
*2	30	*M*	(*a*) 143
			(*b*) 200
3	30	*M*	314
4	24	*F*	180
*5	28	*M*	(*a*) 180
			(*b*) 227
			(*c*) 300
6	18	*F*	200
7	25	*M*	333
8	25	*M*	250
9	23	*F*	231
10	21	*F*	170
11	55	*M*	260
12	35	*M*	200
13	30	*M*	200
14	28	*M*	215
15	23	*M*	139
			Average 219

* The figures *a*, *b* and *c* were obtained on subjects 2 and 5 on different days.

Effect of exercise on the level of AHG activity in the blood of mildly affected haemophilic subjects

It having been observed that strenuous exercise raises the AHG activity of the blood of normal subjects, experiments were carried out to see if haemophilic subjects would react to exercise in a similar manner. Only mildly affected haemophiliacs took part in these experiments, the risk of joint and muscle injury in severely affected haemophiliacs being too great to justify their taking part in such an investigation.

Three experiments were carried out on two haemophilic volunteers. On the basis of family history, severity of bleeding and level of AHG in their blood, both were considered to be mild cases (Biggs & Macfarlane, 1958). Samples of blood were taken before and after a $\frac{3}{4}$ mile run and the level of AHG activity assayed. In each case there was a marked increase in the AHG activity after exercise, the level after exercise being approximately 250% of the level before (Table 2). The increase in activity is thus of the same order as that obtained in the normal subjects.

TABLE 2. Effect of exercise on the plasma AHG level of mildly affected haemophilic subjects

Subject	Age	AHG level (% of normal plasma)		AHG level after exercise (% of level before exercise)
		Before exercise	After exercise	
A.T.	22	(*a*) 25	60	240
		(*b*) 30	70	233
J.B.	26	15	40	266
				Average 246

Lines (*a*) and (*b*) refer to two runs by A.T. on different days.

Possibility of artifact

Effect of exercise on levels of other clotting factors. It is possible that the increase of AHG in the blood recorded after exercise is an artifact and is due to the assay system being sensitive to changes in some other plasma constituent. In the assay method used in this study the test plasma was adsorbed with $Al(OH)_3$ and then various dilutions of the plasma were tested in a modified thromboplastin generation test. It has been found in this test that the amount of thromboplastin generated is directly proportional to the amount of AHG present, provided the other clotting factors are kept constant. It was therefore essential to study the level of other clotting factors after exercise, especially those factors known to take part in the generation of thromboplastin. In experiments on two normal subjects the levels of Christmas factor, Factor V, prothrombin, fibrinogen and AHG were assayed before and after exercise. The level of AHG activity rose in both subjects, but there was no increase in any of the other factors studied (Table 3).

Possible effect of fibrinolysin on the AHG assay. Biggs *et al.* (1947) have shown that strenuous exercise produces fibrinolytic activity in the blood of normal human beings. In the present study all the subjects who undertook vigorous exercise showed marked fibrinolytic activity in their blood as well as a rise in AHG activity, whereas those subjects who performed less vigorous exercise, a 3 mile and 6 mile walk, for instance, showed little or no increase in fibrinolytic or AHG activity. These findings suggested that the presence of fibrinolysin in the blood might be affecting the AHG

assay and giving falsely high results. This possibility seemed unlikely, however, when it was found that blood collected 4 and 6 hr after exercise still showed high AHG levels (see p. 129) but no detectable fibrinolytic activity. Moreover, in experiments in which streptokinase was added to fresh citrated blood to activate the fibrinolytic system, no rise in AHG activity could be demonstrated. On the contrary, there was a loss of AHG activity.

Infusion into haemophiliacs of plasma from exercised donors. If plasma collected after exercise contains 2–3 times the normal amount of AHG one would expect that an infusion of this plasma into a haemophiliac would produce a greater rise in his blood AHG level than would an infusion of plasma from non-exercised donors. Two severely affected haemophiliacs,

TABLE 3. Effect of exercise on level of AHG and other clotting factors in the plasma of two normal subjects

	Level after exercise (% of level before exercise)	
	Subject 1	Subject 2
AHG	275	250
Factor V	100	100
Prothrombin	105	108
Christmas factor	70	100
Fibrinogen	108	108

F.E. and R.B., were each given 1 litre of plasma prepared from the blood of donors who had performed strenuous exercise. Samples of blood were taken from the two subjects immediately before and immediately after the infusion and then 24 hr later. The AHG response of patient F.E. was consistent with his plasma dose containing 200% of AHG, but in the case of R.B. the response was more than would have been expected from plasma in the dose which he was given. This is so far unexplained. The blood of both patients contained 10% of AHG 24 hr after the infusion (Table 4).

Three days before the above experiment was carried out R.B. was given 1 l. of plasma collected from non-exercised donors. The AHG content of this plasma dose was 80% of normal plasma and the patient's AHG level immediately after the infusion had risen to 12% of normal. Twenty-four hours later the level had returned to zero.

Strictly speaking, these experiments served mainly as a check on the AHG level of the administered plasma. The best evidence for increase in AHG activity would be the demonstration that plasma from exercised donors was more effective than plasma from non-exercised donors in controlling haemophilic bleeding. It would be difficult, however, without an extensive clinical trial to differentiate between plasma doses containing 100 and 200% of AHG respectively.

Effect of exercise on other tests of blood clotting

Several workers have found that the clotting time of blood is shortened by exercise (Hartman, 1927; Mills & Necheles, 1928; Schneider & Zangari, 1951). In view of these reports of increased coagulability of the blood after exercise, experiments were done in which the AHG assay was carried out in parallel with some routinely used tests of clotting function. Five normal subjects and two mildly affected haemophiliacs took part in this investigation. The tests carried out in addition to the AHG assay were: the whole blood clotting time in glass and siliconed tubes, the recalcification time of platelet-rich and platelet-poor plasma in glass and siliconed tubes, the Quick one-stage test and the thromboplastin-generation test. In the case of the two haemophiliacs the prothrombin consumption index was also determined. In none of the tests on normal subjects did the blood or plasma consistently show more rapid clotting after exercise, although the

TABLE 4. AHG response of haemophilic subjects following infusions of plasma from exercised and non-exercised donors

	AHG levels (expressed as % of normal plasma)		
	Plasma dose prepared from blood of exercised donors		Plasma dose prepared from blood of non-exercised donors
	R.B.	F.E.	R.B.
AHG activity in patients' blood before dose	0	0	0
AHG activity of dose	152	200	80
AHG activity in patients' blood after dose	50	25	12
AHG activity in patients' blood 24 hr after dose	10	10	0

level of AHG activity in every case rose to more than 200% of the level before exercise. With regard to the tests in the two haemophilic subjects, the whole-blood clotting time, recalcification time and thromboplastin-generation test were normal before exercise, and showed no change after exercise, but the prothrombin consumption index in both was found to be slightly abnormal before exercise, 23·6 and 16%, and normal after exercise, 9·2 and 7·1%, respectively. (In this laboratory, at present, the upper limit of 'normal' for the prothrombin consumption index is 10%.)

DISCUSSION

In the past the effect of exercise on the blood-clotting mechanism has received little attention. The present investigation shows that strenuous muscular exercise brings about a marked increase of AHG activity in the blood of normal subjects and mildly affected haemophiliacs.

Hartman (1927) found that cat's blood clotted more quickly after exercise and Mills & Necheles (1928), experimenting on human beings and

dogs, also found more rapid clotting after exercise. More recently Schneider & Zangari (1951) have shown that the clotting time of blood in silicone-coated tubes is accelerated after exercise. In the light of the present study it seemed possible that the raised coagulability of the blood after exercise observed by these workers might be related to the increase of AHG activity. In a series of experiments carried out to investigate this possibility it was found that even with an increase of AHG to 250 % of normal there was no convincing evidence that blood clotted more rapidly after exercise.

The finding that the AHG level is increased by exercise is of practical and theoretical importance. Several workers consider that the AHG content of the blood is genetically determined and fixed for the individual (Graham, McLendon & Brinkhous, 1953; Brinkhous, Langdell, Penick, Graham & Wagner, 1954). Although it is possibly true that each individual has a basal level of AHG which is genetically determined, there seems little doubt from the above experiments that the level is not fixed but is labile. This is of considerable practical importance with regard to the AHG assay. In this assay, as in other biological assays involving impure systems, the provision of a suitable standard presents a problem. Biggs (1957) used a pool of four plasma samples to represent 100 % of AHG, whereas others (Pool & Robinson, 1959) used plasma samples obtained from the same individuals at intervals and stored at $-20°$C. Biggs *et al.* (1955) used a freeze-dried concentrate of bovine AHG as a standard and this, in view of the effect of exercise in the level of AHG, would seem to be the most satisfactory standard, especially when one wishes to compare the AHG levels of individuals from day to day.

The mechanism whereby exercise produces a rise in the AHG activity of the blood is not known, but further study of this phenomenon may lead to the understanding of the conditions which govern the level of AHG in the blood of normal people and may throw more light on the nature of the coagulation defect in haemophilia.

SUMMARY

1. Strenuous physical exercise increased the AHG activity of the blood of normal people and of mildly affected haemophiliacs. The rise in AHG activity was apparent after 4–6 min of vigorous exertion and persisted for at least 6 hr.

2. There was no increase in the levels of Christmas factor, Factor V, prothrombin or fibrinogen with exercise.

3. Plasma from exercised donors was infused into two severely affected haemophiliacs. The AHG response in the blood of the haemophilic sub-

jects confirmed that plasma after exercise contained increased AHG activity as reflected in the assay.

4. The high AHG level after exercise was not associated with increased coagulability of the blood in the routinely used blood clotting tests.

I should like to thank Dr R. G. Macfarlane, F.R.S. and Dr Rosemary Biggs for encouragement and advice throughout this work. I am also indebted to the volunteers for their co-operation in the experiments, and to the Medical Research Council for a Clinical Research Fellowship.

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FURTHER STUDIES ON PROTEINURIA IN THE NEW-BORN CALF

BY A. E. PIERCE

From the A.R.C. Institute of Animal Physiology, Babraham, Cambridge

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A few hours after the commencement of suckling the new-born calf acquires a passive immunity which is associated with the appearance of a well defined component in the serum, electrophoretically similar to that of the immune lactoglobulin in the colostrum (see Pierce, 1955). Other proteins, in addition to immune lactoglobulin, can be shown by electrophoretic analysis in the first milkings post-partum, notably casein and β-lactoglobulin; and Deutsch & Smith (1957), Bangham, Ingram, Roy, Shillam & Terry (1958) and Pierce & Johnson (1960) have shown that the mucosa of the intestine is not so selective that proteins in addition to the immune lactoglobulin may not be absorbed.

In studies on the proteinuria of the new-born calf, first reported by Langstein & Neuberg (1907) and further studied by Smith & Little (1924) and Howe (1924), Pierce (1959) suggested that the low molecular weight proteins in the colostrum (Pedersen, 1936; Johnson & Pierce, 1959) were absorbed with the immune lactoglobulin and were subsequently cleared from the circulation by the kidney. The transient proteinuria apparently depended upon the permeability of the intestine rather than on any special characteristics of the kidney with regard to protein clearance. In further studies immunological, electrophoretic and ultracentrifugal evidence was obtained which identified most of the urinary protein with that in the colostrum, particularly the β-lactoglobulin, rather than with the pre-colostral serum protein (Pierce & Johnson, 1960; Pierce, 1960). The present experiments examine the permeability of the kidney of the new-born calf to proteins absorbed from the gut, with particular reference to their molecular weight. The β-lactoglobulins of colostrum, molecular weight 35,400–40,000 (see McMeekin, 1954), have been replaced with gelatin, polydisperse, but with a molecular weight of the same order of magnitude (Scatchard, Oncley, Williams & Brown, 1944), and fed to new-born calves. By the use of this simplified system the permeability of the gut to a protein other than immune lactoglobulin has been confirmed and its significance in the aetiology of proteinuria examined.

METHODS

Care and management of calves. The management of the calves (Shorthorn or Jersey) has already been described (Pierce, 1959). In experiments where calves were deprived of colostrum, streptomycin was given as a prophylactic against intercurrent infection.

Electrophoresis was carried out in a Perkin–Elmer apparatus (Tiselius, 1937), with phosphate buffer pH 8·0, ionic strength, $I = 0{\cdot}2$. The procedure adopted for routine runs, and the method of analysis and of mobility measurement have already been described (Pierce, 1959).

Protein fractionation. Albumin and fetuin. The method of preparation of these fractions has already been described (Pierce, 1959).

Immune lactoglobulin and β-lactoglobulin. The casein was precipitated from bovine colostrum at 37° C with rennin. The immune lactoglobulin or β-lactoglobulin component was recovered after electrophoretic separation of the whey proteins from the appropriate limb of the U tube with a fine Pasteur pipette.

Urine proteins. Urine proteins were separated electrophoretically and the required components recovered from the U tube.

Gelatin and γ-globulin. A commercial gelatin product (Hopkins & Williams, London) was used for feeding experiments, and the same material after centrifugation, dialysis and sterilization was used for intravenous injection. The bovine γ-globulin was supplied by Armour Laboratories, U.S.A.

Protein estimations. Protein concentrations were calculated from micro-Kjeldahl nitrogen determinations on duplicate samples using a protein:nitrogen ratio of 6·25. No allowance was made for non-protein nitrogen in serum or colostrum samples. Urine samples were extensively dialysed before analysis.

Ultra-violet absorption. The ultra-violet absorption, E_{1cm} at 276 mμ for the various protein solutions, was determined in quartz cells by means of a Unicam spectrophotometer SP 500.

RESULTS

Calves fed on colostrum

Five calves (E 17, P 1, R 50, S 3, S 8) fed on colostrum showed a rising rate of protein excretion from birth to 14–27 hr (phase *a*). The permeability of the intestinal mucosa to protein macromolecules was confirmed by the electrophoretic analysis of the serum, which showed a steadily increasing concentration of passively acquired immune lactoglobulin for all calves over this period. Intestinal permeability was assumed to be decreasing from the time when, although colostrum was still being fed, there was no further rise in the level of serum immune lactoglobulin. This latter period coincided with a rapid fall in the rate of protein excretion (phase *b*) to levels of less than 1·0 mg/min.

The rate of protein excretion in the urine from calf R 50 shown in Fig. 1*a* is typical and was obtained by feeding 500 ml. of colostrum containing 5·8 g total protein or 3·9 g whey protein/100 ml. every 3 hr over the whole experimental period from shortly after birth to 56 hr 48 min.

The rapid rise in the serum level of immune lactoglobulin from 0·24 g at birth to 1·1 g/100 ml. at 30 hr 50 min is shown in Fig. 2*a*; the total serum protein increased from 4·5 to 4·7 g/100 ml. over the same period.

The electrophoretic pattern of the serum at 26 hr 30 min after birth shows the presence of a well-defined γ-globulin component (Fig. 5*f*).

The pattern of protein excretion rate for calf R 50 may be compared with that for calf S 19 (Fig. 1*b*) which was given 500 ml. of 0·15 M-NaCl containing 5 g glucose/100 ml. every 4 hr from birth to 46 hr 15 min, and thereafter colostrum at the same rate for a further 8 hr. At no time did the excretion rate exceed 1 mg/min; this level and lower is also shown by normal calves fed with colostrum after the period of proteinuria and will be referred to as normal for the young calf. The electrophoretic analysis of the serum showed that no immune lactoglobulin had been absorbed 9 hr 40 min after the beginning of colostrum feeding (Fig. 2*b*).

TABLE 1. Data relating to injections given to calves P 13, E 19, E 20 (Shorthorns) and D 7 (Jersey) maintained on glucose-saline

Calf			Serum injected					Electrophoretic analysis			
			Vol.*						Globulins		
Number	Weight (kg)		I.V. (ml.)	S.C. (ml.)	Injection (no.)	Period after birth+ (hr)	Concentration (g/100 ml.)	Albumin	α	β	γ
P 13	41·8	Pre-colostral	815	25	8	2·0–35	5·2	51	35	11	3
E 19	—		200	150	7	3·5–48	—	55	31	11	3
E 20	—	Post-colostral	700	250	8	3·5–44	6·0	52	23	9	16
D 7	21·8		785	0	5	6·0–37	10·2	31	28	11	30

+ Time to nearest ½ hr. — No value. * I.V., intravenous; S.C., subcutaneous.

Calves injected intravenously with pre- or post-colostral calf serum

An attempt was made to examine the permeability of the kidney in the new-born calf to rising concentrations of normal calf serum proteins. The protein excretion rates were determined for three new-born calves (P 13, E 19 and E 20) injected with large volumes of undiluted pre- or post-colostral serum derived from other new-born calves. The injected calves were maintained on glucose-saline given in feeds of 500 or 1000 ml.

Although the total volume of serum injected was large (for details see Table 1), the serum protein level in the recipient calves showed a decrease. Therefore the experiment failed, since the rise in total serum protein which occurred over the first 48 hr in those calves fed on colostrum could not be reproduced. In a further attempt to raise the plasma protein concentration a fourth calf, D 7, was fed on glucose-saline and injected intravenously during the first 37 hr of life with 785 ml. of post-colostral calf serum which had been previously concentrated to 10·2 % protein (approximately $\times 2$). The serum protein level again fell from 4·7 to 3·95 % during the first 9 hr of life and only slowly regained the concentration at birth by the 42nd hour, rising further to 5·1 % by the 48th hour. The steady rise in γ-globulin

derived from the injected post-colostral calf serum is shown at Fig. 2*c*. The rate of protein excretion for this calf, in common with P 13, E 19 and E 20, remained within the normal limits, not exceeding 1·0 mg/min (Fig. 1*c*).

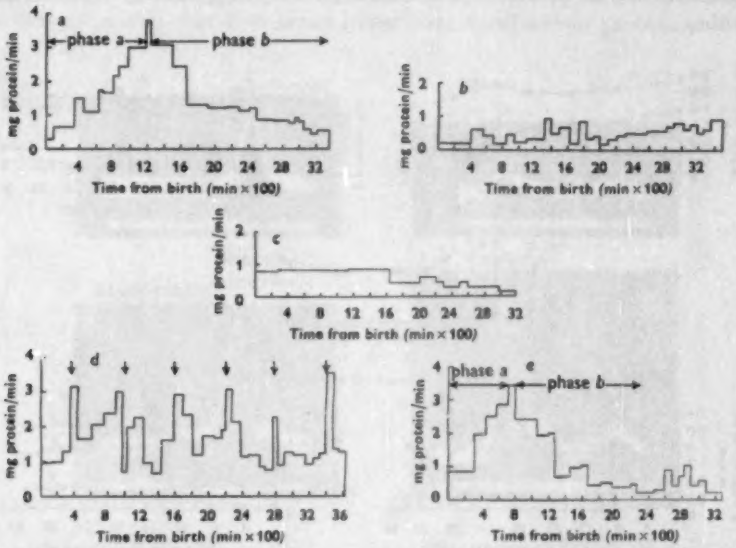

Fig. 1. The urinary protein excretion rates from birth for calves fed on (*a*) colostrum, calf R 50; (*b*) saline, S 19; (*c*) gelatin, R 17; and injected intravenously with (*c*) post-colostral calf serum, D 7, and (*d*) gelatin, E 10, injection at each arrow. Note time unit, 100 min.

Calves injected intravenously with gelatin

The electrophoretic mobility together with the low U.V. absorption characteristic for gelatin have been used to identify this protein in the urine. The electrophoretic and ultracentrifuge patterns for the gelatin used for feeding and intravenous injection are shown in Fig. 3*f*, *g*. The gelatin migrated electrophoretically as a single component with a mobility $\approx 1{\cdot}9\ \mathrm{cm^2/V.sec} \times 10^{-5}$, which is similar to immune lactoglobulin, and sedimented as a spreading peak indicating polydispersity. Taking the peak maximum as the position of the boundary, a sedimentation constant $s^0_{20} \approx 1{\cdot}44\ S$ was obtained. Figure 4 shows the U.V. absorption at $\lambda = 276$ for gelatin, pre-colostral calf serum, serum albumin, fetuin and γ-globulin, β-lactoglobulin and immune lactoglobulin. The electrophoretic patterns of these proteins are shown in Fig. 3.

The calculated regression lines for γ-globulin and immune lactoglobulin

were almost identical and the difference was not significant. Those for serum albumin and β-lactoglobulin were identical. The U.V. absorption for fetuin was low compared with the other serum proteins, and fetuin and albumin form the major components of pre-colostral serum, so that the regression line for precolostral calf serum lies between that for albumin and fetuin.

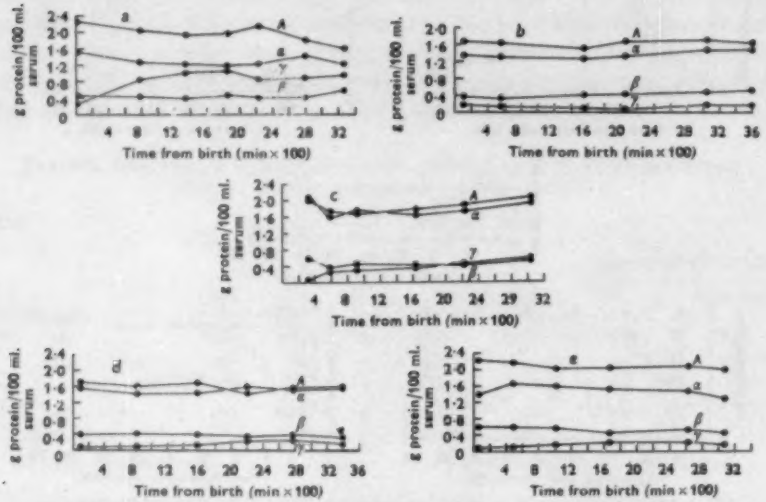

Fig. 2. The sequence of changes from birth in the electrophoretic components of sera for calves fed on (*a*) colostrum, calf R 50; (*b*) saline, S 19; (*c*) gelatin, R 17; and injected intravenously with (*c*) post-colostral calf serum, D 7, and (*d*) gelatin, E 10. In this and subsequent figures A = albumin, α = α-globulin, β = β-globulin, γ = γ-globulin.

Calf E 10 was deprived of colostrum and fed on heat-sterilized milk at a constant rate (500 ml. every 4 hr) during the first 56 hr of life. At intervals of 10 hr (6 × 100 min) 20 ml. of 0·15 M-NaCl containing 3 g gelatin was injected into the jugular vein. The bladder was emptied shortly before each injection. Figure 1*d* shows the rise in the protein excretion rate which followed each intravenous injection. The gelatin would migrate with the γ-globulin component, but Fig. 2*d* shows only a very slight rise of doubtful significance over the period of the injections. The electrophoretic analysis of urine collected at 58 hr (0·2 g protein/100 ml.) showed that the component with the mobility of gelatin (1·9 $cm^2/V.sec \times 10^{-5}$) contributed 82·5% of the protein.

Urine derived from calves during the proteinuria which followed intravenous injection of gelatin gave U.V. absorption values lower than serum

protein of comparable concentration. For example, calf S 20 was fed on glucose-saline and showed a U.V. absorption for dialysed urine, collected 115 min after the intravenous injection of 3 g gelatin when 9·5 hr old, 1·52 times lower than pre-colostral serum protein. The slow component fractionated by electrophoretic separation and with a mobility comparable to that of gelatin was 2·85 times lower than total serum protein or 4·06

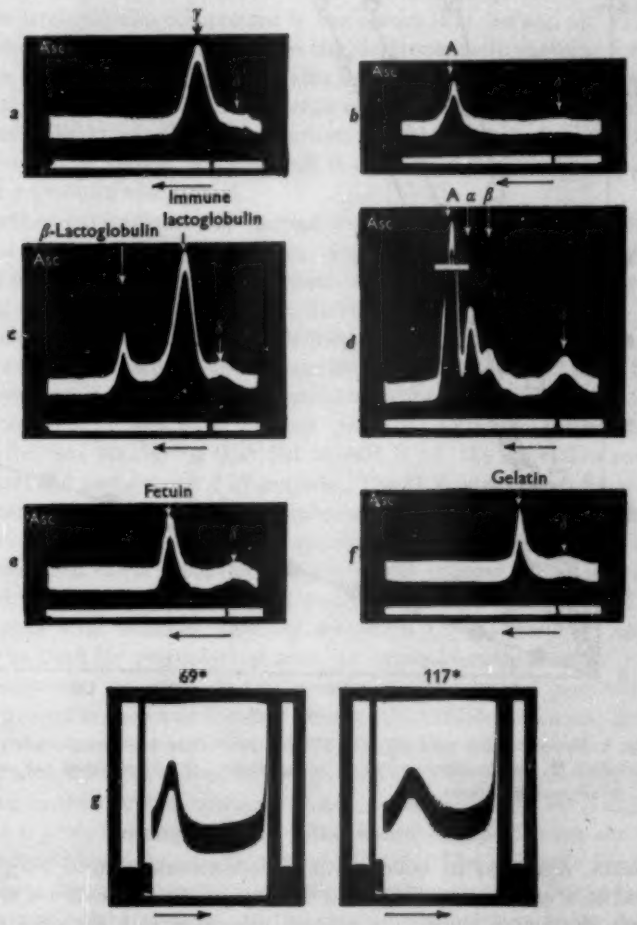

Fig. 3. The ascending electrophoretic pattern in phosphate buffer pH 8·0, $I = 0·2$ for (*a*) serum γ-globulin; (*b*) serum albumin; (*c*) colostral whey; (*d*) precolostral calf serum; (*e*) serum fetuin; (*f*) gelatin, and (*g*) ultracentrifuge pattern for gelatin in phosphate buffer pH 8·0, $I = 0·2$; * time in min at 59,780 rev/min.

times lower than immune lactoglobulin. The electrophoretic pattern for this urine is shown in Fig. 5*b* and may be compared with that for calf E 25 (Fig. 5*a*) which was fed on colostrum. Most of the urinary protein for E 25 had a fast mobility while the injected calf had a major slow component within the mobility range for gelatin.

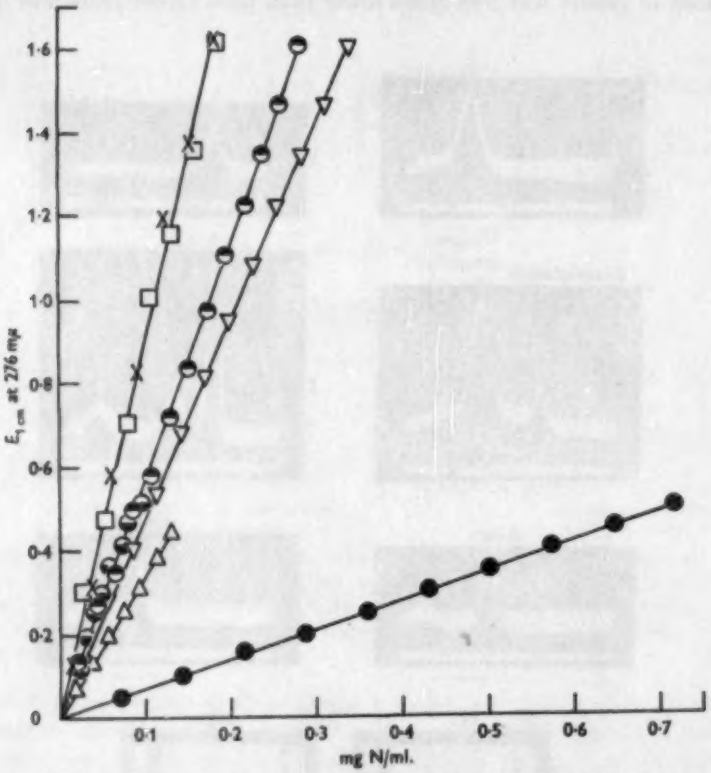

Fig. 4. The regression lines ($E_{1\,cm}$ at 276 mμ) for X, immune lactoglobulin; □, γ-globulin; ◓, serum albumin; ◒, β-lactoglobulin; ▽, precolostral calf serum; △, fetuin and ●, gelatin.

Calves fed on boiled milk and gelatin

Gelatin, dissolved in boiled milk at a concentration of 4·0 g/100 ml., was fed to a new-born calf R 17 at the rate of 500 ml. every 4 hr. A proteinuria developed showing a protein excretion rate divisible into two phases *a* and *b* and characteristic for calves fed on colostrum. The transition from phase *a* to phase *b* was also within the period associated with the decrease in intestinal permeability (Fig. 1*e*).

The concentration of gelatin was within the normal range for immune lactoglobulin in colostrum and the amount fed over the first 48 hr (240 g) was greater than the immune lactoglobulin fed to R 50 which, over a comparable period, received only 167·1 g. However, the electrophoretic analysis of the serum from R 17 failed to reveal the passive absorption of any significant amount of protein with a mobility comparable with gelatin and this is in sharp contrast to calf R 50 which acquired a well-defined immune lactoglobulin component in the serum (Fig. 2*a* and *e*). Figure 5*e* and *f* shows the difference between the electrophoretic patterns for serum proteins from calf R 17, fed on gelatin and for calf R 50 fed on colostrum. In the former case there is no evidence of a passively acquired component with the mobility of gelatin, the pattern resembling that shown at Fig. 3*d* for pre-colostral serum, whereas calf R 50 has passively acquired a well-defined γ-globulin component.

A further experiment was carried out in which the boiled milk was replaced by 0·15 M-NaCl containing 4 g gelatin and 5 g glucose/100 ml. and fed to calf G 4 at the rate of 500 ml. every 4 hr. Proteinuria developed showing the characteristic pattern divisible into phases *a* and *b*. Again it was difficult to show any well-defined gelatin component in the serum quantitatively comparable to immune lactoglobulin absorbed after feeding colostrum, but nevertheless a component with the mobility of gelatin formed well over 50% of the total urinary protein. Although the amount of gelatin fed was similar to that fed to calf R 17, the excretion rate was lower and did not exceed 2·27 mg/min. The U.V. absorption for the slow component fractionated by electrophoretic separation from the urine of calf R 17 (fed on gelatin) was low, approximating that for gelatin, and at least 4·5 times lower than for a comparable concentration of immune lactoglobulin, which has a similar electrophoretic mobility, while those components with a faster mobility showed a U.V. absorption approximating to that for pre-colostral sera. A typical electrophoretic pattern for the proteins in urine during proteinuria which had resulted from feeding gelatin is shown in Fig. 5*c*. Most of the protein had a low mobility and may be compared with that for gelatin in Fig. 5*d*.

Urine samples from calves fed on gelatin were divided into two groups according to their protein nitrogen values, those exceeding 0·055 mg N/ml. and with a protein equivalent of 0·172 g/100 ml. dialysed urine are shown in Fig. 6*a* and those below this value in Fig. 6*b*. The values in Fig. 6*a* lie below those for serum protein and deviate towards those for gelatin as the nitrogen values rise, i.e. as the proteinuria becomes more marked, while the values in Fig. 6*b* lie around those for pre-colostral serum protein.

Similar U.V. absorption studies on urine samples from two calves (S 3, S 8) fed on colostrum are shown in Fig. 6*c*, where the protein

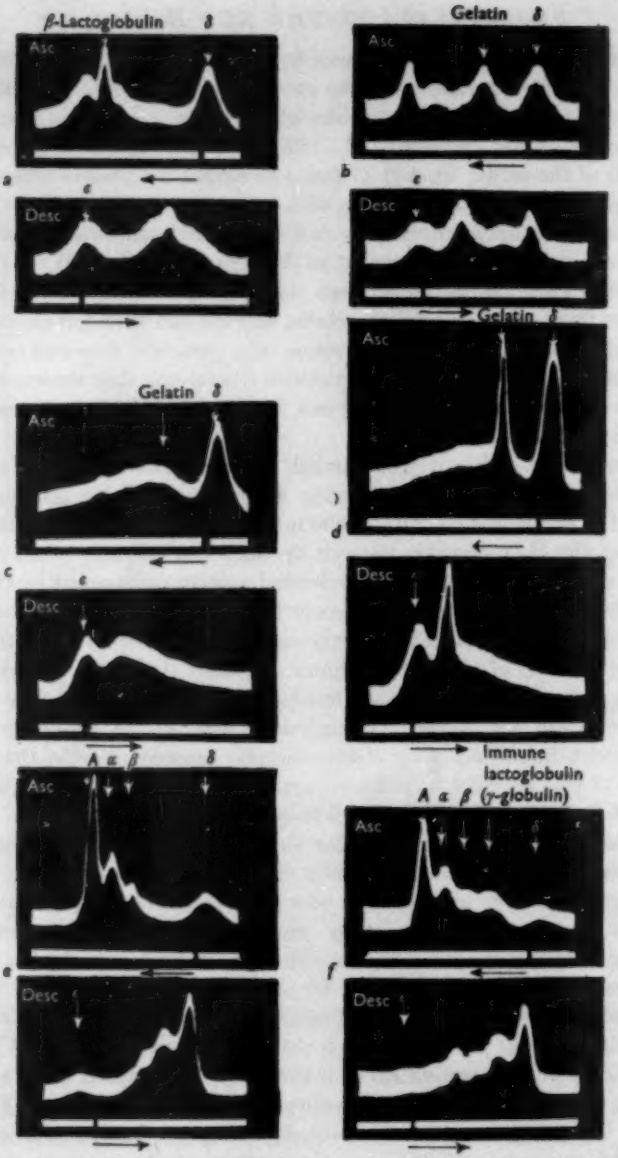

Fig. 5. The electrophoretic patterns in phosphate buffer pH 8·0, $I = 0{\cdot}2$ for the urine proteins during proteinuria from calves (*a*) fed on colostrum, calf E 25; (*b*) fed on saline and injected intravenously with gelatin, S 20 (3 g/20 ml. saline); (*c*) fed on gelatin in 0·15 M-NaCl, F 4; and (*d*) urine from F 4 to which gelatin has been added: also the electrophoretic patterns for serum proteins; (*e*) 27 hr 40 min after feeding on gelatin, R 17, and (*f*) 26 hr 30 min after feeding on colostrum, R 50.

equivalence per milligram nitrogen is 0·688 g/100 ml. dialysed urine. These values were generally higher than those for pre-colostral serum protein.

The electrophoretic fractionation of the protein in a urine sample from

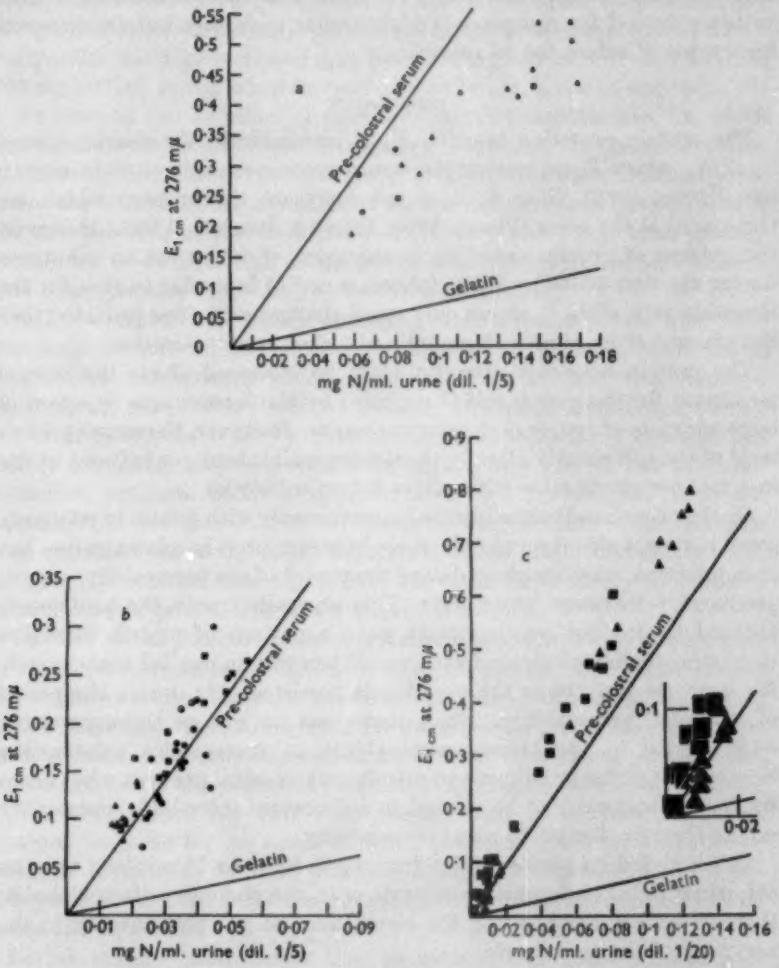

Fig. 6. U.V. absorption $E_{1\,cm}$ at 276 mμ for urine protein from calves R 17 (▲), F 4 (■), and R 80 (●) fed on gelatin: (*a*) during proteinuria when values exceeded 1·7 mg protein/ml. urine, and (*b*) when values were less than 1·7 mg protein/ml. urine; *a*, *b* urine diluted 1/5: and (*c*) for urine (diluted 1/20) from calves S 3 (▲), and S 8 (■) fed on colostrum during phases *a* and *b* of proteinuria (see p. 137). Inset on double scale.

a calf fed on colostrum showed that the major component with the mobility of β-lactoglobulin approximated to the U.V. absorption for albumin or β-lactoglobulin. The remaining slower components gave a higher value, between those for albumin and γ-globulin, and may be contrasted with values obtained for components with similar mobilities but fractionated from urine of calves fed on gelatin.

DISCUSSION

The protein excretion rate ($U_p.V$) is preferable to the clearance rate, $U_p.V/S_p$, where S_p represents the serum protein concentration in mg/ml. (see Pierce, 1959), since S_p does not represent the proteins which are eliminated in the urine (Pierce, 1960; Pierce & Johnson, 1960). However, the pattern of protein excretion in the urine of calves fed on colostrum during the first 50–56 hr of life (phases *a* and *b*) is similar to that for the clearance rate, since S_p shows only small changes with time provided that the amount of immune lactogiobulin absorbed is not excessive.

The protein excretion rate could not be increased above the normal maximum for the young calf (1 mg/min) by the intravenous injection of large amounts of pre- or post-colostral serum. However, the serum protein level of the calf shortly after birth was not maintained nor induced to rise in a manner comparable with calves fed on colostrum.

On the other hand calves injected intravenously with gelatin in relatively small amounts also showed a decrease in serum protein concentration but each injection, whether given during the period of gut permeability or not, produced a transient proteinuria. This contrasted with the proteinuria induced by feeding gelatin, which gave a pattern of protein excretion characteristic for colostrum-fed calves. When gelatin was fed continuously for approximately 56 hr the proteinuria persisted only during the period of intestinal permeability. Since there was no change throughout the 56 hr period in the kidney permeability to intravenous gelatin, the termination of the proteinuria to gelatin and colostral proteins when given by mouth appeared to be related to a decreased intestinal permeability rather than to changes in renal permeability.

Calf S 19 fed on glucose saline from birth to 46 hr 15 min and then on colostrum failed to develop proteinuria or to absorb immune lactoglobulin, thereby further emphasizing the significance of gut permeability in the aetiology of the proteinuria.

Although these experiments were not directed towards the question of the selectivity of gut absorption relative to different proteins, it is of interest that when 167 g of immune lactoglobulin was present in the colostrum fed to calf R 50, the serum component with comparable mobility increased from 263 mg/100 ml. 20 min after birth and before feeding to

913 mg/100 ml. when the calf was 46 hr 30 min of age, a rise of 247 %. Since the gelatin and immune lactoglobulin have similar mobilities, one could be detected in the undegraded form in the serum as readily as the other. However, 220 g gelatin fed to calf R 17 failed to produce any comparable rise over a similar period. The serum protein component with a comparable mobility increased only from 204 mg/100 ml. 1 hr after birth, to 223 mg/100 ml. serum when the calf was 44 hr old, a rise of only 9 %.

No account can be taken of metabolic or extravascular loss, for which there could be differences between proteins. However, the protein excreted in the urine, which would be selective for gelatin on the basis of comparative molecular weight, could not account for the differences in the amounts of the two proteins in the circulation; R 17 only lost 3·5 g. total urinary protein up to 44 hr of age, of which the electrophoretic analysis showed that other proteins made an appreciable contribution.

Unless extravascular losses, excluding that into the urine, were substantially different for gelatin than for immune lactoglobulin, these results suggest that the immune lactoglobulin (mol. wt. 180,000) was absorbed more readily than the low mol. wt. gelatin. Brambell, Hemmings, Oakley & Porter (1960) have shown that although undegraded γ-globulin was readily absorbed, after digestion with papain only one of the three degradation products each of approximately 1/3 the original mol. wt. possessed the 'recognition unit' by which the acceptability of the molecule from the maternal to the foetal circulation of the rabbit was determined. In a similar manner, this might offer the basis for a theory of selective transmission of protein across the intestinal mucosa.

The U.V. absorption of the urine proteins during the proteinuria induced by feeding colostrum contrasted with those fed gelatin. In the former case the values exceeded those for pre-colostral serum, while in calves fed on gelatin the values were lower than for pre-colostral serum and tended to deviate further towards the values for gelatin with rising protein concentration, i.e. as the degree of proteinuria increased. In calves deprived of colostrum, when the excretion rate returned to normal the values returned to those for *pre-colostral* serum proteins. Such an observation would be compatible with the presence of partially degraded, non-dialysable products derived from the agammaglobulinaemic serum proteins of calves fed on gelatin.

Earlier studies have shown that in some calves fed on colostrum, degraded immune lactoglobulin but with retained antibody activity could be recovered from the urine during proteinuria (Pierce, 1959; Pierce & Johnson, 1960). The presence of this degraded protein may have contributed to the raised U.V. absorption/mg N obtained with urinary protein from the calves fed on colostrum.

SUMMARY

1. Colostrum, but not glucose-saline, when fed to new-born calves induced a proteinuria. Calculations based on protein excretion rate showed two phases: (*a*) rising values while the gut was permeable to undegraded protein, and (*b*) declining values after the period of gut permeability.

2. A calf fed on glucose-saline for the first 46 hr of life and then on colostrum did not develop a proteinuria and the gut was not permeable to immune lactoglobulin. Feeding colostrum from birth to 55 hr did not prolong the normal period of proteinuria nor that of the gut permeability.

3. New-born calves fed on sterilized milk or glucose-saline and injected intravenously with gelatin developed a transient proteinuria after injections given during and after the period of gut permeability.

4. New-born calves fed on gelatin in glucose-saline developed a proteinuria showing a protein excretion rate characteristic for calves fed on colostrum; gelatin frequently provided over 50% of the protein excreted.

5. The results of the electrophoretic analysis of the serum proteins suggested that immune lactoglobulin in colostrum was more readily absorbed than gelatin.

6. The proteinuria of the new-born suckling calf does not appear to be related to any special permeability of the kidney to protein, but to the permeability of the gut to undegraded proteins such as gelatin and the β-lactoglobulin component of colostrum, which have similar molecular weights *ca.* 40,000, and are below the threshold for glomerular filtration.

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THE MAXIMUM LENGTH FOR CONTRACTION IN VERTEBRATE STRIATED MUSCLE

By A. F. HUXLEY* and L. D. PEACHEY†
From the Physiological Laboratory, University of Cambridge

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In their well-known experiments on isolated muscle fibres, Ramsey & Street (1940) showed that the rise of tension during an isometric tetanus decreases linearly as the length is increased beyond a certain value l_0 near to the resting length, reaching zero when the fibre is stretched to about twice this length. A. F. Huxley & Niedergerke (1954) pointed out that a simple explanation for this result was given by the sliding-filament theory of muscular contraction put forward by them and by H. E. Huxley (1953; H. E. Huxley & Hanson, 1954), if it is supposed that a relative force between filaments of the two sets is generated at each of a number of sites in the zone where the two sets of filaments overlap. If these sites are distributed evenly, the total tension generated should be proportional to the length of the overlap zone, which should decrease linearly as the sarcomere length is increased from about 2·0 μ (the length of the secondary filaments), reaching zero at a sarcomere length of about 3·5 μ (the sum of the lengths of the primary (about 1·5 μ) and of the secondary filaments).

There were, however, several uncertainties about this explanation, as follows.

(1) Ramsey & Street had not measured striation spacing in their fibres, so it was not clear exactly what the sarcomere length was when the developed tension just fell to zero. From their definition of l_0 it did nevertheless seem likely that the tension reached zero only if the fibre was stretched to a sarcomere length appreciably greater than 3·5 μ.

(2) Ramsey & Street had found that in these highly stretched fibres the tension during an isometric tetanus rose in two stages, the first with about the same speed as in less stretched fibres, the second being very much slower. The quick phase formed a progressively smaller proportion of the total tension rise as the fibre was stretched, and reached zero at a considerably lesser degree of stretch than the slow phase. It was not clear which of these end-points ought to be used.

* Present address: Department of Physiology, University College London.
† Present address: Department of Zoology, Columbia University, New York 27, U.S.A.

(3) No accurate determination of filament lengths had been made, so it was not possible to say beyond what sarcomere length there was no overlap between the two sets of filaments.

The slow component in the rise of tension in stretched muscle was also observed by Hill (1953) in whole sartorius muscles of frogs and toads. He suggested that the explanation might be that those parts of the muscle which happened to be less extended than others would generate more tension, and would shorten, and stretch the more extended parts, so increasing any initial inequality in the degree of extension. The tension would rise slowly as this redistribution of length took place. Fischer (1926) showed that the passive extensibility of the sartorius muscle is less near its ends than in the middle, so that the redistribution of length in an isometric tetanus of a highly stretched muscle might take place by shortening of the ends, with corresponding elongation of the middle.

Preliminary experiments showed that the slow phase of tension increase in isolated fibres during an isometric tetanus does arise in this way and that isometric recording did not give a sharp end-point. We therefore measured the maximum sarcomere length at which contraction occurs, by mounting a fibre isotonically and detecting shortening by microscopic observation of the striation spacing in its middle part. In these experiments the ends of the fibre were able to shorten without raising the tension in the middle of the fibre, and we were thus able to distinguish between shortening of the ends of the fibre and that of the middle. The filament lengths in the muscle used were determined in two ways by electron microscopy.

The results (excluding the measurements by electron microscopy) have already been reported briefly (Huxley & Peachey, 1959).

METHODS

Dissection

Twitch fibres were isolated from the dorsal head of the semitendinosus muscle (and in a few cases from the iliofibularis muscle) of the frog *Rana temporaria*. The muscle was initially divided with fine scissors, and the fibres subsequently separated by cutting with a knife the collagen fibrils which unite them. The muscles were immersed during the dissection in Ringer's solution with the addition of tubocurarine (10^{-6} g/ml.) at room temperature.

Solutions

The Ringer's solution used both in the dissection and for the experiments themselves had the following composition (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85 (pH 7·2).

Stimulation and recording

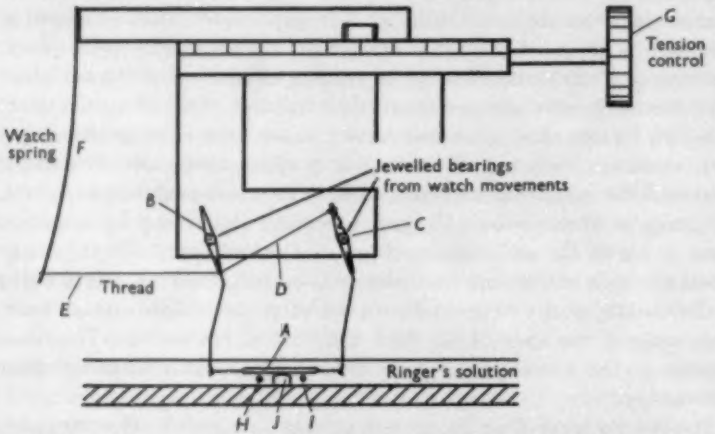
The fibre was immersed in Ringer's solution in a channel 3 mm broad and 3 mm deep. In the isotonic experiments a piece of glass rod 1·5 mm diameter was laid across the bottom of this channel, and the fibre was so placed that it rested lightly on the surface of this rod

at a point which did not move appreciably on stimulation. The stimulating electrodes were Pt wires which ran across the bottom of the channel, 4 or 6 mm apart, one each side of the glass rod. Since the fibres were 10–20 mm long, the stimulating electrodes were well within the length of the fibres. For tetanic stimulation alternating condenser discharges of about 1·7 msec time constant at either 40 or 50/sec were employed.

An RCA 5734 electromechanical transducer valve was used for isometric recording; it was mounted on a screw-driven slide with a vernier scale for adjusting the length of the fibre.

An isotonic device was constructed from the balance wheels of two watches, with their jewelled bearings. Their movements were linked by a thread, as shown in Text-fig. 1, so that a point near the middle of the fibre would remain stationary during shortening of the fibre. The over-all shortening was not recorded, but changes of striation spacing in the part of the fibre which lay on the glass rod were recorded by cine micrography.

All experiments were carried out at room temperature (17–20° C approx.).



Text-fig. 1. Diagram of isotonic lever system. The fibre *A* is held between two levers of stainless-steel wire attached to the cut-down balance wheels *B* and *C* of two watch movements. These are linked by a thread *D* which ensures that they turn in opposite directions, so that some point within the length of the fibre stays stationary during shortening. The ratio of the amounts of movement permitted at the two ends can be altered by hooking *D* on to different notches on *C*. Tension is applied through the thread *E* from a piece of watch mainspring *F*, and is adjusted by moving the fixed end of the spring by the knob *G*. The levers lie in an oblique plane so that the microscope objective (not shown) can come down vertically above the fibre. *H*, *I*, stimulating electrodes; *J*, glass rod supporting the fibre.

Microscopy

The trough was mounted on the mechanical stage of a microscope so that any point on the fibre could be brought into the field. The fibre was observed by direct microscopy, using a Zeiss D* water-immersion objective, focal length 4·4 mm, n.a. 0·75, which dipped directly into the Ringer's solution. The metal surrounding the front lens was coated before each experiment with 'paraffin and rubber lubricant' (British Drug Houses Ltd.) to prevent metallic ions from entering the solution and damaging the fibre. The n.a. of the illuminating cone was about 0·25. Still photographs were taken with a 35 mm reflex camera, using Ilford

Pan F film and an over-all magnification of 138× or 360×. Cine photographs were taken, generally at 55 frames/sec, on Ilford 16 mm FP3 film, with an over-all magnification of 63 ×. A low-voltage tungsten lamp was used, with a green filter.

Determination of filament lengths

Bundles of 30–50 fibres were dissected from semitendinosus muscles of frogs, with the same procedure as in the early stages of preparing a single fibre (the procedure eliminates the region of the nerve entry, where slow fibres are found).

Sections. Four bundles were used; they were stretched to give striation spacings of 3·0–3·8 μ in the middle parts of the fibres. The length of each bundle was measured between two threads tied around the tendons as near as possible to the ends of the muscle fibres. The bundles were fixed while held at these lengths in the trough by running in a fixative with the following composition: 1% OsO_4, 2% sucrose, 0·01 M-$CaCl_2$ buffered to pH 7·4 with 0·035 M sodium acetate and 0·035 M sodium veronal. After 40 min fixation at room temperature, the ends of the bundles were detached and their lengths measured again. The bundles were then dehydrated through a series of ethanol–water mixtures and lengths were measured again in absolute ethanol. Each bundle was cut transversely into four pieces and the length of each piece measured, still in absolute ethanol. These pieces were then divided into two groups, the two proximal pieces of each bundle for methacrylate embedding and the two distal pieces for embedding in Vestopal-W (Ryter & Kellenberger, 1958). All pieces were stained overnight in 1% phosphotungstic acid in ethanol and then transferred through the various solutions for impregnation with the respective embedding media. The length of each piece was measured in its final liquid embedding medium and again after the blocks had been hardened for approximately 15 hr in a 60° C oven.

One shrinkage factor was determined from the above measurements for each bundle from fixation to absolute ethanol, and another for each piece from absolute ethanol to the final embedded block. These two factors were combined to give an over-all shrinkage factor for each piece.

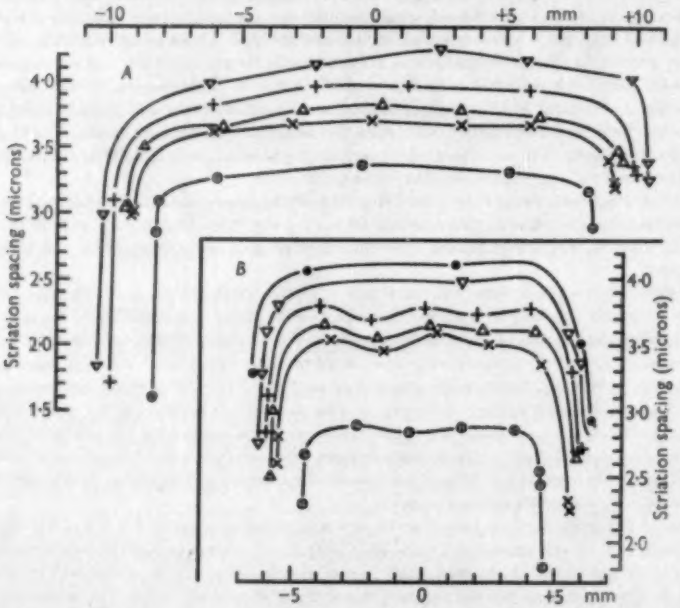
Longitudinal sections were cut on either a Porter–Blum or an A. F. Huxley (1957*a*) microtome, with the long axis of the fibres perpendicular to the direction of movement in order to avoid alteration of filament lengths from compression of the sections. The sections were mounted on grids covered with carbon films, and low power electron micrographs were taken in either a Siemens Elmiskop I or an RCA EMU-2B electron microscope. The magnification of each electron micrograph was determined accurately by photographing the same area of the grid under the light microscope and measuring the distance between features that could be identified in both images. Measurements for magnification determination and measurements of filament length in the electron micrographs were made on the negatives with a travelling microscope.

Fibrils. Each bundle was stretched to give a striation spacing of 2·7–2·8 μ, flooded with formol-calcium (1 vol. formalin, 1 vol. 10% $CaCl_2$, 8 vol. water) and left several hours in the fixative. It was then cut up into small pieces, suspended in 50 ml. water and treated for 1 min in a homogenizer (Measuring and Scientific Instruments Ltd.). The suspension was centrifuged (1600 *g*) for 1 min, the supernatant discarded, the precipitate resuspended in 50 ml. water and treated in the homogenizer for three further periods of 10 min each, with cooling in between. It was centrifuged again to remove large pieces, the supernatant transferred to fresh tubes and centrifuged for 1 hr. The sediment was resuspended in a total volume of 1 ml. A drop of this suspension was placed on an electron microscope specimen grid with a formvar film on the underside of which a layer of carbon had been deposited. Thin fibrils were selected under the electron microscope (Siemens Elmiskop I) and micrographs were made at a magnification of about 2000. The exact magnification was determined in the same way as with the sections. Measurements were made on the negatives with a travelling microscope.

RESULTS

Experiments on living muscle

Non-uniformity of striation spacing in stretched fibres. The striation spacing was measured by photography with the 35 mm camera, at a series of positions along two isolated muscle fibres. In each case it was found that the spacing near the ends of the fibre was substantially smaller than in the middle; this is illustrated by the micrographs reproduced in Pl. 1, figs. 1 and 2. The variation of striation spacing along the fibres at several degrees of stretch is shown graphically in Text-fig. 2. It is seen that the spacing varies little except within about a millimetre of each end of the fibre, where it drops sharply. Isolated measurements made visually with a



Text-fig. 2. Variation of striation spacing along isolated muscle fibres at rest. *A*, fibre from semitendinosus muscle (15. xi. 58); *B*, fibre from iliofibularis muscle (8. xi. 58). Abscissa: distance from centre of fibre; ordinate: striation spacing, measured on photomicrographs. The various curves for each fibre were obtained at different degrees of elongation. Each point gives the mean value of the striation spacing found by measuring several (usually three or four) rows of 15–30 striations at different parts of the same negative, except in a few cases near the ends of the fibres where the striations were partly obscured by overlying fibrous tissue, and could only be measured in one or two small fields.

micrometer eyepiece showed a similar drop at the ends of several other fibres, all of which were considerably stretched. In no case was the drop absent.

Electron micrographs of sections taken near to one end of an isolated fibre are reproduced in Pl. 3, figs. 1 and 2. The sarcomere lengths are 2·7 and 3·1 μ and the two sets of filaments overlap considerably, although in the middle part of the same fibre the sarcomere length was about 3·8 μ.

At the extreme left-hand end of Text-fig. 2*A* three of the graphs drop to spacings below 2 μ while the other two only reach down to about 3·0 μ. All these measurements were made at the extreme end of the fibre, so that the difference cannot be due to observing slightly different parts of the fibre. In the cases where the spacing is short, the striations are very irregular, and the fibre had been stimulated tetanically just before it was photographed. It is thus probable that this end of the fibre had entered the 'delta state' (Ramsey & Street, 1940), and had not yet stretched out when these micrographs were taken.

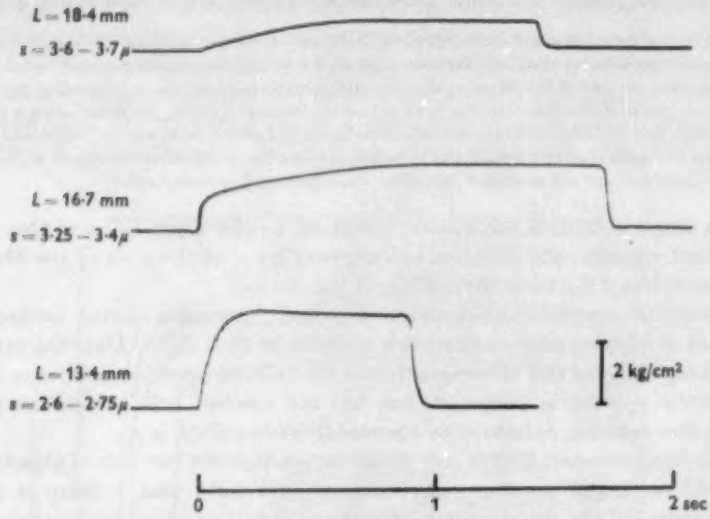
A single complete toe muscle (extensor longus digiti IV) was also examined visually; the striation spacing was 2·6 μ at the ends of the fibres when it was 3·6 μ near the middle of the muscle.

Isometric contractions. A series of records of tension during isometric tetani at varying degrees of stretch is shown in Text-fig. 3. Over the range investigated, the rise of tension during the tetanus becomes smaller as the striation spacing is increased, but has not reached zero even when the striation spacing, measured in the middle of the fibre, is 4 μ.

At this sarcomere length one would expect that the two sets of filaments would no longer overlap, suggesting at first sight that overlap is not necessary for the development of tension. An alternative explanation is, however, provided by the observation described in the preceding section, since the uppermost curves in Text-fig. 2*A* and 2*B* show that even when the striation spacing over the main part of a fibre is 4 μ, at the ends it may be well under 3·5 μ, roughly the maximum value at which overlap could be expected. The tension rise may therefore be due to shortening of the ends of the fibre without any active contribution from those parts where the spacing exceeds 3·5 μ, as was suggested in the introduction. This explanation is further suggested by the time course of the tension rise. In agreement with the observations of Ramsey & Street (1940) and Hill (1953), this contains a slow component which becomes progressively more conspicuous as the fibre is stretched to greater lengths. As Hill pointed out, this is what would be expected if one part of the fibre were shortening at the expense of other parts.

It was found by microscopic observation of the ends of the fibre that such a shortening does take place. Plate 1, figs. 3 and 4, show micrographs of the end of a fibre stretched so that the striation spacing in the middle was 3·9–4·2 μ, taken before (fig. 3) and during (fig. 4) tetanic stimulation.

A high degree of shortening is seen although the fibre is held at both ends so that its total length remained constant to within a few microns. Both ends were found to shorten in this way, so that the middle part of the fibre must have been stretched. Cine-micrographs of the middle of the fibre showed little change apart from slight longitudinal movement; the increase of sarcomere length expected is rather small, as only a small fraction



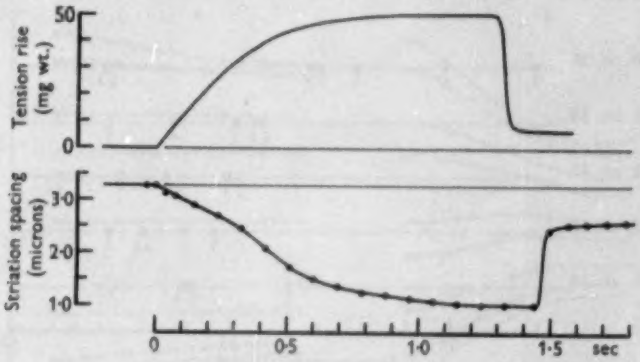
Text-fig. 3. Records of tension during isometric tetani at three lengths. Semitendinosus fibre; same experiment as Text-fig. 2*A*; top record corresponds to points +, and middle record to points ⊙, in that diagram. The values for the sarcomere length *s* refer to the main part of the length of the fibre, the value being substantially lower at the ends. The scale for tension per unit area is calculated from the cross-section at a striation spacing of 2·5 μ.

of the fibre length at each end shortens. The fact that only the ends shortened cannot be attributed to a failure of propagation, since the two stimulating electrodes were fairly close together near the middle of the fibre, and the action potentials must have propagated over a distance of several millimetres in order to reach the ends.

Text-figure 4, lower curve, shows the time course of the shortening, found by measurement of the cine-micrographs. It is seen to agree fairly well with the time course of the tension rise, which is plotted in the upper curve.

If the slow component is attributed on this evidence to shortening at the ends, the fast component should be due to active tension developed along the whole length of the fibre, and should disappear if the sarcomere length

in the main part of the fibre reaches a value at which no tension is developed. Text-figure 3 shows that the fast component is not recognizable when the sarcomere length in the middle is above about 3·6 μ, but its disappearance is not sharp enough to allow a precise determination of the limit to be made. In the same way the isometric twitch became very small when the main part of the fibre was stretched to a sarcomere length above about 3·8 μ, but again a sharp end-point was not found, presumably because of shortening at the ends.



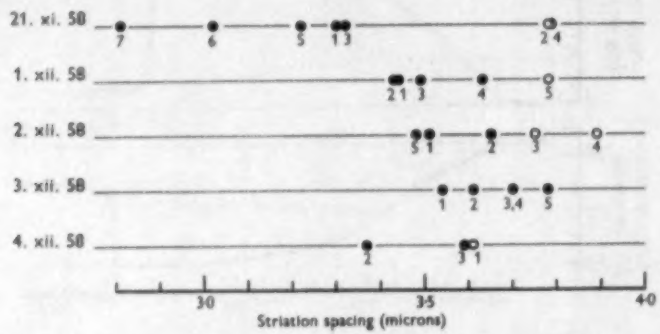
Text-fig. 4. Time course of shortening near one end (lower curve) and tension rise (upper curve), recorded during isometric tetani. Same fibre as Text-figs. 2*A* and 3; successive tetani at length corresponding to points marked △ in Text-fig. 2*A*. Shortening obtained from measurements on cine film.

Experiments on toe muscles. Two attempts were made to observe this shortening near the ends in a whole muscle stimulated at great length. The extensor longus digiti IV of the frog was used. Both experiments failed because the muscles broke. In one case this occurred when the striation spacing in the middle was about 3·3 μ; in the other it had reached 3·6 μ, but the muscle broke before a record could be obtained.

Isotonic experiments. To obtain a result unaffected by shortening at the ends, fibres were held under constant tension in the apparatus shown in Text-fig. 1 and shortening of the middle part of the fibre was recorded by cine-micrography. Changes in total length were not recorded and shortening of the ends was immaterial, since the isotonic lever prevented length changes from altering the tension to which the middle part of the fibre was subjected.

Three types of result were observed: (1) no change apart from slight longitudinal movement, (2) vigorous shortening, (3) slow, delayed, irregular shortening, occurring only in patches within the field of view.

These are illustrated in Pl. 2, and the conditions under which they were observed in the various experiments are shown in Text-fig. 5. It will be seen that vigorous shortening was always obtained if the striation spacing before stimulation was 3·5 μ or less, and that at 3·54 μ or more no shortening occurred apart from the delayed, patchy shortening which was seen in a few cases, chiefly in fibres which had already been stimulated at a length where vigorous shortening just occurred. In run 1 of the experiment of 3. xii. 58, where the initial value of the striation spacing was 3·54 μ, the spacing did not begin to fall until more than 0·2 sec after stimulation was begun.

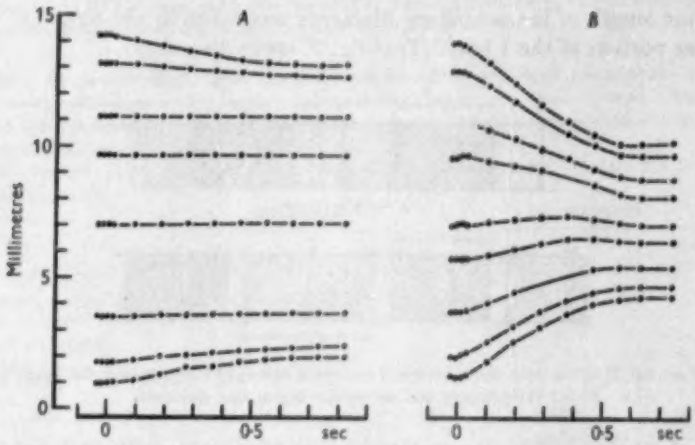


Text-fig. 5. Results of tetanic stimulation of fibres held isotonically. ●, vigorous shortening; ○, no shortening; ⊕, delayed, patchy shortening. Abscissa: striation spacing immediately before stimulation, measured on the cine film. Five experiments, all on fibres from the semitendinosus muscle. The numbers indicate the order in which the tetani at different initial lengths were carried out.

In a single experiment a different method of recording was used. The fibre was held by the same isotonic lever system, but its whole length was photographed with the cine camera (55 frames/sec; over-all magnification 0·5 ×). Short pieces of thread were placed on the fibre to act as markers. The striation spacing near the middle of the fibre was measured with the water-immersion objective and a micrometer eyepiece. The fibre was first stimulated (40/sec) at a striation spacing of 3·6 μ; both ends of the fibre shortened but at least the middle 60 % was unchanged. The tension was then reduced, giving a spacing of 3·4 μ; the whole of the fibre now shortened on stimulation, the middle part decreasing to a little below half its unstimulated length. The length changes of the various segments of the fibre in the two cases are plotted in Text-fig. 6.

Conclusions from experiments on living fibres. It seems fair to conclude from these experiments that no contraction occurred in parts of fibres where the striation spacing exceeded a critical value which we estimated at

3·52 μ (limits of error 3·50–3·54 μ). The irregular shortening sometimes seen when the measured spacing was slightly above this value may reasonably be attributed to contraction which began in small areas where the spacing was below the critical figure, and spread slowly as laterally adjacent areas became passively shortened. This process would greatly exaggerate any pre-existing differences of spacing; the resulting irregularities were clearly visible on relaxation and did not disappear at once, which would explain the fact that this type of response was seen more frequently in



Text-fig. 6. Shortening of different segments of a fibre during isotonic tetani. Fibre from iliofibularis muscle. Initial striation spacing near middle of fibre was 3·6 μ in *A*, 3·4 μ in *B*. Top and bottom lines represent the ends of the fibre; intermediate lines show positions of pieces of thread adhering to the fibre. Measurements made on selected frames of the low-magnification cine film, taken at 55 frames/sec.

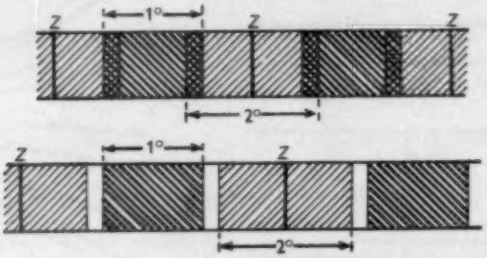
fibres which had already been stimulated at lengths close to the critical value. The only experiment where this type of behaviour was seen at spacings more than 3% above our estimate of the critical value was that of 3. xii. 58; in and after run 3 of that date the cine film shows differences of spacing of that order before stimulation even within its rather limited field of view.

Measurement of filament lengths by electron microscopy

Method of measurement. The method of measurement of filament lengths was in accordance with the arrangement of primary and secondary filaments demonstrated by H. E. Huxley (1957). The length of the A band was taken, in each case, as the length of the primary filaments. In cases where

the sarcomere length was such that the two sets of filaments overlapped (Pl. 3, figs. 1, 2 and 4), the length of the secondary filaments was taken as the length of the I band plus the sum of the lengths of the adjacent dark overlap zones of the two neighbouring A bands (Text-fig. 7, upper diagram).

In cases where the sarcomeres were stretched so far that there was no overlap and the two sets of filaments were 'out of mesh' (Pl. 3, fig. 3), the A bands contain no outer dark zone, but the I bands contain an outer pale zone. This region was taken to be a gap between the two sets of filaments and the length of the secondary filaments was taken as the length of the darker portion of the I band (Text-fig. 7, lower diagram).



Text-fig. 7. Diagrams showing the dimensions taken as representing the lengths of the primary and secondary filaments. See text.

Embedded material. A sample field from one of the embedded preparations used for filament length determination is illustrated in Pl. 3, fig. 3. The measurements of filament lengths are summarized in Table 1. The final mean value for the sum of the lengths of the primary and secondary filaments is 3·52 μ, which is the same as the value found in our isotonic experiments for the maximum sarcomere length at which contraction occurs.

The final values in Table 1 have been corrected by shrinkage factors determined for each embedding medium as described under Methods; the distribution of the shrinkage between the various stages of the embedding procedures is given in Table 2. These factors are for shrinkage from the fixed bundle after release of tension to the final embedded pieces. Shortening during fixation and on release of tension has been neglected; it was small (0, 0, and 1·6 %) except in the case of one bundle which appeared to lose 10·8 % of its length in this step. Since this bundle closely paralleled the others in all subsequent stages and gave final results in good agreement with the others, it seems likely that the original measurement of length was incorrect in this one case.

These shrinkage factors have been applied to the sum of the measured lengths of the two kinds of filaments, a procedure which is precisely valid only if both filaments shrink by the same percentage of their lengths. It is not obviously true that this is the case; indeed it seems quite possible that A and I bands might shrink by different amounts, and for this

reason the correction for shrinkage has not been applied to the two types of filament separately. However, on any reasonable assumption about the mode of shrinkage, there is no error in the corrected value of the sum of the filament lengths if the sarcomere length is such that the two sets of filaments just do not overlap, and the error will be of opposite sign according as the sarcomere length is greater or less than this value. In our material the average sarcomere length was close to this value, so that the error of the final mean should be small. As an example, we recalculated the data on the assumption that all the shrinkage took place in the I bands, the A bands including the overlap zones remaining constant. The resulting value for the sum of the filament lengths for the methacrylate specimens was increased by 0·017 μ from that obtained by the procedure used in Table 1; that for the Vestopal-W specimens was decreased by 0·021 μ; and there was no change in the second decimal place for the mean of the two values. It does not seem likely that non-uniformity of shrinkage could introduce an error of more than a few hundredths of a micron.

TABLE 1. Measurements of filament lengths in sections from embedded preparations. Values in microns, without correction for shrinkage except in the last row. Each value for a filament length is the mean of five measurements on the negative. All calculations carried to at least one extra decimal place before rounding.

The striation spacings measured on the four bundles before fixation were 3·8, 3·5, 3·0 and 3·7 μ respectively

	Methacrylate					Vestopal-W				
Bundle no.	1	2	3	4	S.E. of means	1	2	3	4	S.E. of means
Striation spacing in area measured	3·18	3·09	2·41	3·71	—	3·48	2·57	2·77	3·14	—
Filaments lengths										
Primary	1·45	1·48	1·40	1·46	0·015*	1·48	1·44	1·42	1·49	0·025*
Secondary	1·73	1·73	1·73	1·72	0·023*	1·70	1·72	1·70	1·70	0·017*
Sum	3·19	3·21	3·13	3·18	0·028†	3·17	3·16	3·12	3·20	0·031†
Mean of sums		3·176			0·016‡		3·160			0·016‡
Mean over-all shrinkage (%)		9·05			—		10·78			—
Sum of filament lengths, corrected for shrinkage		3·492			0·017		3·542			0·018

* Standard error of mean from any one bundle, estimated from the combined variance between individual measurements on each bundle.

† Square root of sum of squares of values for primary and secondary filaments.

‡ Standard error of combined mean estimated from the differences between the 'sum' values for the four bundles.

TABLE 2. Longitudinal shrinkage (%) at various stages of embedding in methacrylate and in Vestopal-W. Each value is the mean of the shortening during the corresponding stage expressed as a percentage of the length of the material lying free in the fixative. The figures in brackets give the number of measurements on which the values are based

Stage		Longitudinal shrinkage	
From	To	Methacrylate	Vestopal-W
Free in fixative	Ethanol	5·91 (4)	
Ethanol	Embedding liquid	1·55 (7)	4·12 (8)
Embedding liquid	Block	1·59 (7)	0·75 (8)
Combined		9·05	10·78

There is no significant correlation between sarcomere length and the small variations in the measured filament lengths. This is illustrated both by the values in Table 1 and by the micrographs reproduced in Pl. 3.

Separated fibrils. Plate 3, fig. 4, shows an electron micrograph of a separated fibril, typical of the ones on which measurements were made. Fibrils were selected in the field of view solely on the criteria of having a straight stretch several sarcomeres long and of being thin enough for the band pattern to be well seen. Two preparations gave the following values for the filament lengths (in microns): primary filaments, $1{\cdot}516 \pm 0{\cdot}010$ (S.E. of mean from 7 fibrils) and $1{\cdot}492 \pm 0{\cdot}011$ (8); secondary filaments, $1{\cdot}918 \pm 0{\cdot}009$ (7) and $1{\cdot}923 \pm 0{\cdot}020$ (8). The pairs of results do not differ significantly from one another, and the combined value for the sum of the lengths of the two filaments is $3{\cdot}425 \pm 0{\cdot}013\ \mu$.

These preparations were in distilled water for about 4 hr, from the time when the disintegration was begun till the droplet was dried down on the grid. Two other preparations in which the fibril suspension in water was kept in one case for 2 and in the other for 3 days before making the grids gave lower values for both sets of filaments (means $1{\cdot}477\ \mu$ for primary and $1{\cdot}858\ \mu$ for secondary filaments, sum $3{\cdot}335\ \mu$). Presumably shrinkage had occurred, and these values are therefore discarded. This result does suggest the possibility that even the value obtained from the fibrils which had not spent a long period in water is slightly low on account of shrinkage.

As in the embedded preparations, there was no correlation between sarcomere length and filament lengths. However, only a rather narrow range was covered, most of the sarcomere lengths falling between $2{\cdot}5$ and $2{\cdot}8\ \mu$.

DISCUSSION

The electron microscope observations provide further evidence that passive length changes take place by a sliding motion of one set of filaments relative to the other, the changes in filament length being within experimental error and in any case not more than a small percentage of the changes in sarcomere length. Since most of our measurements on sections were made at striation spacings near the point where overlap ceases, any small change of filament length with stretch will alter only slightly our estimate of the critical length. Allowance for the amount of change apparently present would raise our estimate from $3{\cdot}52$ to $3{\cdot}54\ \mu$. The measurements on separated fibrils are less reliable since there is a suggestion that some shrinkage had occurred and there was no means of allowing for it; nevertheless, the value obtained for the sum of the filament lengths, $3{\cdot}43\ \mu$, is only 3% below that derived from the sections, and this is probably as good an agreement as could be expected.

The experiments on living isolated fibres were carried out on the same type of muscle (twitch fibres of m. semitendinosus of the frog) as was used in the electron-microscope work, and showed that contraction does not occur when the sarcomere length exceeds 3·52 μ. The agreement between this figure and the electron-microscope value for the point where the filaments cease to overlap suggests strongly that contraction can occur only if there is an overlap between the two sets of filaments. This is probably the first actual evidence that contraction depends on an interaction between the filaments in the region where they overlap, as postulated in all forms of the sliding-filament theory. It does not, however, throw any light on the nature of this interaction, or help to eliminate any of the particular systems that have been proposed (A. F. Huxley, 1957*b*; Weber, 1958; Podolsky, 1959; Spencer & Worthington, 1960).

The observation that during tetanic stimulation a high degree of shortening can occur at the ends of a fibre whose total length is held constant is an example of the instability which, as Hill (1953) pointed out, results from the negative slope of the isometric length–tension diagram of highly stretched muscle. The delayed, irregular shortening that was sometimes seen in fibres stretched to a point where normal contraction had disappeared is probably another way in which this instability can show up. These observations emphasize the need, in experiments on stretched muscle, for checking with the microscope whether contraction is taking place uniformly along the length of the muscle.

SUMMARY

1. In isolated striated muscle fibres of the frog under stretch the striation spacing was found to be substantially lower near the ends than in the main part of their length.

2. The slow tension rise during an isometric tetanus of greatly stretched fibres was found to be due to shortening of the ends.

3. In isotonic tetani it was found by cine-micrography that no shortening occurs at points where the striation spacing exceeds a critical value which is close to 3·5 μ.

4. The filament lengths in the same muscle were determined by electron microscopy both on sections and on separated myofibrils. It was found that the two sets of filaments do not overlap if the sarcomere length exceeds 3·5 μ.

5. Contraction thus ceases to occur if a fibre is stretched to such an extent that there is no overlap between the two sets of filaments. This is taken as evidence that contraction depends on an interaction between filaments of the two types in the regions where they overlap, as postulated

in the various theories of contraction based on the idea that length changes take place by a relative sliding motion of the filaments.

These experiments were carried out while one of us (L.D.P.) was a Graduate Fellow of The Rockefeller Institute. We also wish to thank the Wellcome Trust who provided one of the electron microscopes used in this work.

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EXPLANATION OF PLATES

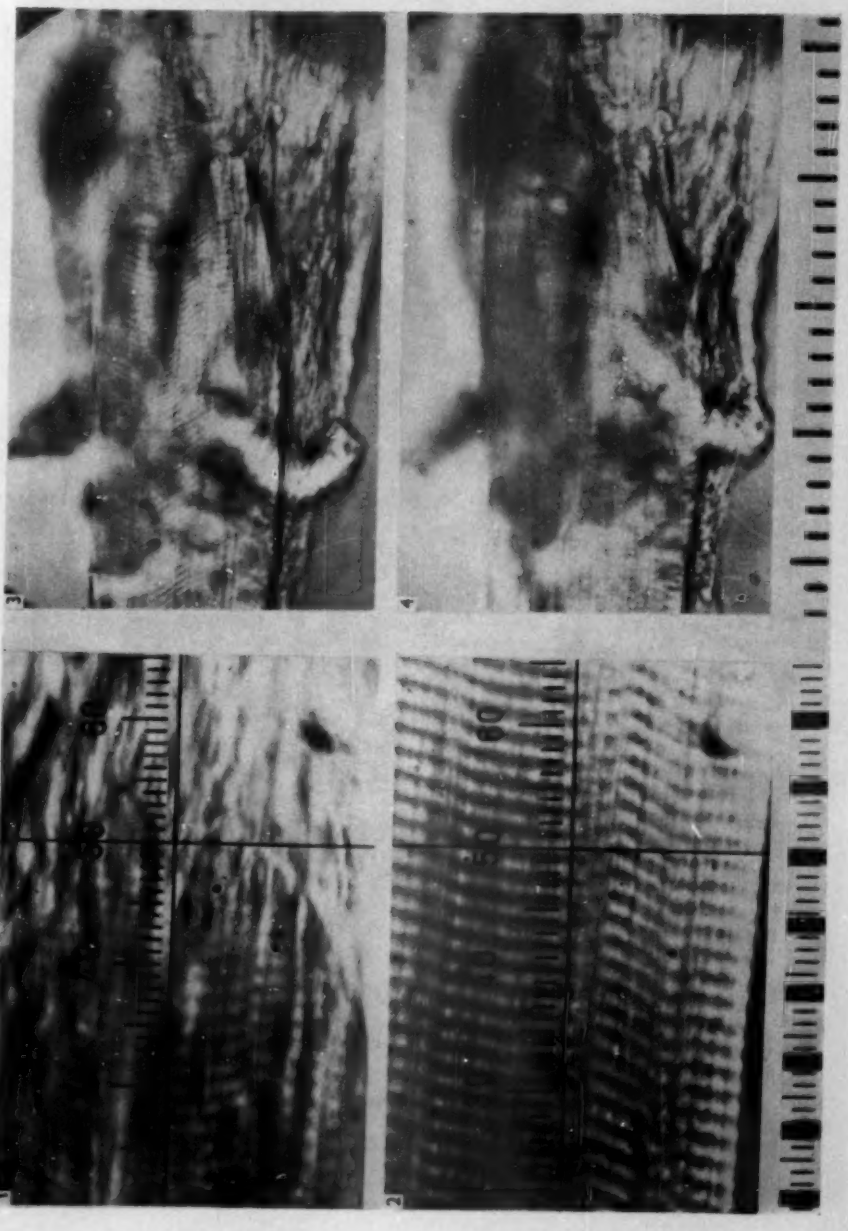
PLATE 1

Figs. 1 and 2: still micrographs showing the difference in striation spacing between the end of a fibre (fig. 1) and a point close to the centre of the same fibre (fig. 2), the over-all length of the fibre being the same in both cases. Striation spacing 2·83 μ in fig. 1; 3·42 μ in fig. 2. Fibre from semitendinosus muscle; prints from same series of exposures as lowest points in Text-fig. 2*A*. Stage micrometer: 10 μ divisions.

Figs. 3 and 4. Shortening at end of a fibre during repetitive stimulation at fixed over-all length. Figure 3 before, fig. 4 about 1 sec after, beginning of stimulation. Sarcomere length in fig. 3, 3·1 μ; sarcomere length in middle region of the fibre was 4·0–4·2 μ. Same fibre as in figs. 1 and 2, but opposite end. Stage micrometer: 10 μ divisions.

PLATE 2

Fibres held under constant tension during tetanic stimulation. Selected frames from cine records, showing the three types of response obtained. In each column the top print is from the last frame before movement due to stimulation can be detected on the film.



A. F. HUXLEY AND L. D. PEACHEY

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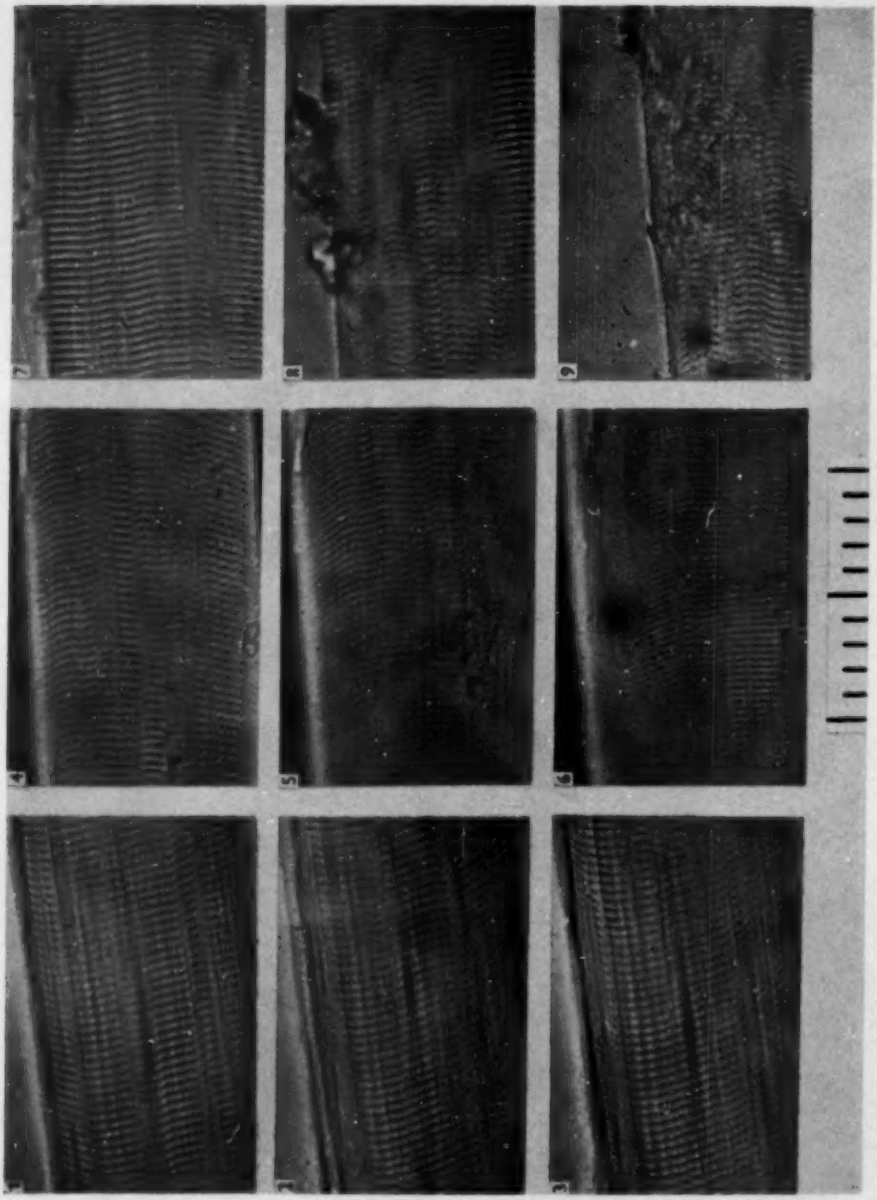

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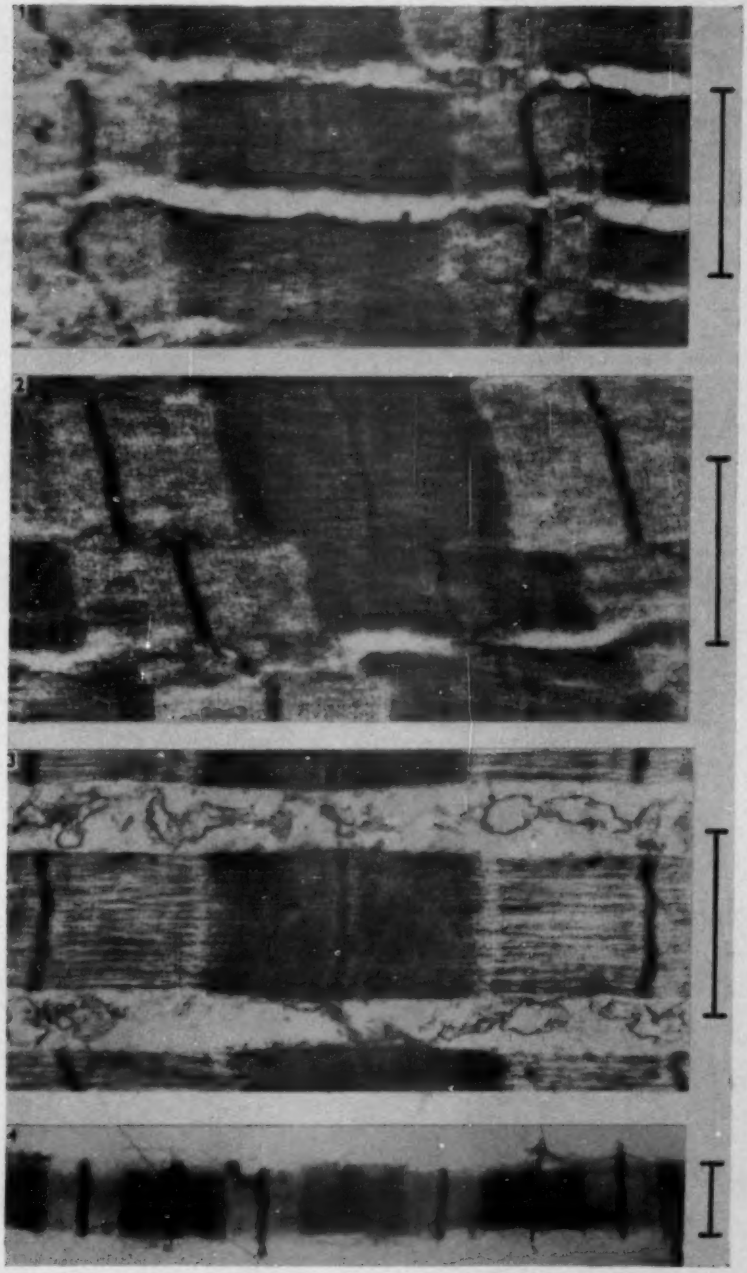

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Figs. 1–3. Run 1 of 4. xii. 58. No shortening at initial striation spacing of 3·61 μ. Figure 1, immediately before stimulation; fig. 2, 1·0 sec later; fig. 3, 2·2 sec after fig. 1. No detectable change in striation spacing.

Figs. 4–6. Run 1 of 2. xii. 58. Rapid shortening at initial striation spacing of 3·51 μ. Figure 4, immediately before stimulation; fig. 5, 0·13 sec later, striation spacing 3·28 μ; fig. 6, 0·24 sec after fig. 4, striation spacing 2·80 μ.

Figs. 7–9. Run 2 of 3. xii. 58. Delayed, irregular shortening at initial striation spacing of 3·61 μ. Figure 7, immediately before stimulation; fig. 8, 1·0 sec later; fig. 9, 2·2 sec after fig. 7. Shortening barely detectable in fig. 8 or in lower left-hand part of fig. 9, but upper right-hand part of fig. 9 shows irregular contraction.

Stage micrometer (10 μ divisions) at same magnification as figs. 1–3 and 7–9; figs. 4–6 are at 1·5% lower magnification.

PLATE 3

Figs. 1–3. Electron micrographs of longitudinal sections of fast striated muscle fibres from the semitendinosus of *Rana temporaria* cut with the long axis of the fibres parallel to the knife edge. Section thickness, 60–80 mμ. Scale bars, 1 micron.

Fig. 1. Section about 40 μ from the end of the isolated fibre used in the experiment of 3. xii. 58, showing a sarcomere length of 2·7 μ and an overlap of about 0·5 μ (corrected values) even though the centre of the fibre was stretched to a sarcomere length of about 3·8 μ. Fibre fixed and embedded in methacrylate as described in the text, except that fixation was for only 15 min. Magnification, × 28000.

Fig. 2. Section about 400 μ from the end of the same fibre as in fig. 1, showing somewhat less overlap (about 0·2 μ) and a sarcomere length of 3·1 μ (corrected values). Magnification, × 28000.

Fig. 3. Section from a bundle of fibres embedded in Vestopal-W showing a sarcomere stretched to a length (3·7 μ, corrected value) slightly greater than the critical length, with the primary and secondary filaments 'out of mesh'. The suggestion of fine filaments connecting the ends of the two major sets of filaments may be a result of superposition in the section or may represent an additional component of the myofibril. Magnification, × 28000.

Fig. 4. Electron micrograph of a separated myofibril representative of those on which measurements of filament lengths were made. Magnification, × 11000.

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DARK-ADAPTATION AND THE REGENERATION OF RHODOPSIN

By W. A. H. RUSHTON*

From the Ophthalmology Branch, National Institutes of Health, Bethesda, Md., U.S.A.

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Ever since Kohlrausch (1922) measured dark-adaptation by plotting the logarithm of the threshold against the time in the dark, it has been clear that the recovery of visual sensitivity occurs in two stages. For, in general, the curve plotted exhibits two quasi-independent branches which meet at a pretty sharp kink. As Kohlrausch demonstrated, the early and faster process is associated with day vision: the later process with night vision. An enormous amount of subsequent work has substantiated this interpretation, but we are on much less secure grounds if we conclude (as is often done) that the first branch is concerned with cones only, the second with rods only and that there is no rod–cone interaction.

The 'cone branch' is obtainable from the rod-free fovea; it has the spectral sensitivity of daylight vision, monochromatic test flashes are usually seen as coloured, acuity is good, and a large Stiles–Crawford effect is found. This is strong evidence that cones are involved, but weak evidence that rods are not involved. To be sure, they are not involved at the central fovea, but the cone branch there is not quite the same shape as that found at the parafovea, where rods are anatomically present (Hecht, Haig & Wald, 1935; Sloan, 1950).

The 'rod branch' is obtainable from all parts of the retina where rods are present and is absent from the only places (fovea and blind spot) where they are not. This branch exhibits the spectral sensitivity of twilight vision which (after correction for absorption by the ocular media) corresponds very closely to the absorption spectrum of rhodopsin (Crescitelli & Dartnall, 1953), a photosensitive pigment which can be seen contained in the living rods (Boll, 1876). Test flashes appear colourless whatever their wave-length, acuity is poor and the Stiles–Crawford effect is small or absent.

This is strong proof that rods are involved, but evidence is not so clear whether cones contribute or not. If rhodopsin is the only pigment which absorbs quanta at threshold throughout the second branch, then after any

* Present address: Physiological Laboratory, University of Cambridge.

fixed bleach, the curves obtained with test flashes of various wave-lengths should all coincide when suitably displaced parallel to the axis of log. *I*. Such coincidence, however, cannot be obtained from some curves which appear reliable. Kohlrausch (1931) and Wolf & Zigler (1955) found that adaptation with a blue test proceeded faster and more extensively than with an orange test—as might be expected if cones made some contribution to the excitability of the second branch.

On the other hand Sloan (1958), who alternated the colour of the test flash (blue/white) throughout the course of each run (instead of making separate runs for each colour tested), found no change in relative threshold. Sloan's is clearly the surer method of detecting any change in relative sensitivity. We have employed it, using alternately blue and yellow or green and yellow tests and looking for any variation in the differential sensitivity during the course of rod adaptation. We confirm Sloan in finding an exact equality throughout the second branch of the curve and conclude that, in the conditions of our experiments, cones enter no more into any part of this branch than they do at the final stage of complete dark-adaptation.

Actually the rod and cone curves do not meet quite sharply at a kink. Almost all published results show a rounded transition lasting a minute or so, as might be expected from the *probability summation* of two independent inputs (Stiles, 1949, p. 223). But the creeping transition which Wald (1960) has recently presented, lasting from the seventh to the seventeenth minute of dark-adaptation, has neither been seen in his earlier publication (Hecht *et al.* 1935; Wald & Clark, 1937), nor in those of other workers; nor was it found in our own experiments, where a very similar technique established a fairly abrupt transition from cones to rods.

It has been widely believed that the fall in threshold exhibited by the rod branch of the dark-adaptation curve represents in some way the regeneration of rhodopsin. Such a relation between threshold and pigment was put forward by Hecht at first to explain the recovery in sensitivity of the marine animals *Mya* and *Ciona*. The analogy with human dark-adaptation led to these ideas being carried over to a domain where exact measurements made them finally unacceptable, and Hecht's (1937) well-known review has only one thing quantitative to say about dark adaptation and the photochemical theory. It is, that the instantaneous threshold in the dark at the end of light-adaptation—the first point upon the dark-adaptation curve—is related to the light level to which the eye had been adapted by a formula which was derived from photochemical considerations and fitted the experiments very well. But the considerations which predicted the first point were quite unable to explain the rest of the curve (as Hecht was well aware), and actually the first point itself was only explicable by assuming that a retinal illumination of 0·03 td will bleach half the rhodopsin away. But this illumination corresponds to the incidence of less

than 1 quantum per rod per second and the direct measurements of Campbell & Rushton (1955) have shown that actually a light one million times stronger is needed. A more detailed discussion of the achievements and difficulties of the photochemical theory has been given elsewhere (Rushton, 1959).

In a determination of the dark-adaptation curve, the actual value of threshold intensity will obviously depend upon the size, duration, wave-length, etc., of the test flash; but if the curve represents simply the regeneration of rhodopsin, its *shape* should remain unaffected by changing these test parameters, since they will produce nothing more than a vertical shift. The observed results, however, turn out to be more complex. For, though change of wave-length may simply result in the vertical shift of the curve (as discussed above) an increase in the area or duration of the flash does increase somewhat the extent of dark-adaptation and prolong the recovery time (Craik & Vernon, 1941; Arden & Weale, 1954). Other factors, therefore, besides the regeneration of rhodopsin must contribute to the increase of excitability during dark-adaptation—presumably a reorganization of nerve connexions, as Lythgoe (1940) pointed out long ago, and as electrophysiology has since confirmed (Barlow, Fitzhugh & Kuffler, 1957*a*, *b*).

The difficulty in establishing securely the photochemical theory has been to measure in the same eye both the visual threshold and the rhodopsin level. For, before the development of retinal densitometry, it was not possible to estimate the visual pigments in man, and in animals not only does the quantitative extraction of rhodopsin need great care and patience, but the concomitant determination of the visual threshold by electrophysiology or behaviour constitutes a difficult and totally different problem. Such measurements, moreover, as were undertaken (Granit, Munsterhjelm & Zewi, 1939; Peskin, 1942) did not suggest any simple or indeed unique relation between rhodopsin content and e.r.g. in the frog. And there was very little evidence indeed to suggest that the increased sensitivity in dark-adaptation was related to the regeneration of rhodopsin, otherwise than through the fact that each represented a return to an equilibrium which had been disturbed by bleaching.

This was the position when retinal densitometry threw some new light upon the relation by permitting the visual pigment level to be measured in the living eye during bleaching and regeneration. It soon appeared that human rhodopsin is more or less completely regenerated by 40 min after the end of a full bleach (Rushton, Campbell, Hagins & Brindley, 1955), whereas human cone pigments are back in about 8 min (Rushton, 1958). Both these times correspond closely to the time needed for return to full visual sensitivity in the dark.

This coincidence strongly suggests that there is some intimate relation between threshold and pigment concentration, for while the pigment is regenerating the threshold is falling, and at about the stage where regeneration stops the improvement in sensitivity also ceases. But is the relation between threshold and pigment unique, and if so what is it? That is the question this paper sets out to answer.

It is known from the work of Hecht and others (Müller, 1931; Hecht, Haig & Chase, 1937; Haig, 1941; Mote, Riopelle & Meyer, 1950) that rod dark-adaptation curves following substantial light-adaptations are all very similar in shape, but are displaced laterally along the time axis to various extents. The greater the bleaching, the more the curve is displaced to the right and the later the recovery at every stage. On the other hand, a more profound rhodopsin bleach will also take longer to reach each fixed stage of pigment regeneration. Are the curves of pigment regeneration also related simply by lateral displacement? And is the time shift the same both for the recovery of threshold and for the regeneration of rhodopsin?

In the experiments to be described the rods were light-adapted to various extents and the subsequent regeneration in the dark was followed by retinal densitometry. The rod thresholds were defined by a single point, namely, the kink in the curve—the moment when the rods first became more sensitive than the cones (always after more than 8 min of dark-adaptation). Thus, after each light-adaptation it was possible in a single run to measure both the curve of pigment regeneration and the moment of cone–rod transition simply by interrupting the former for the short interval required to determine the latter.

It will be shown that no matter how much pigment had to be regenerated ($> 25\%$) the kink always occurred at the moment when 92% had been resynthesized. And in the Discussion it will be argued that this in conjunction with certain well-established facts defines the relation between threshold and rhodopsin content.

METHODS

Dark-adaptation. Records were traced upon Gunkel & Bornschein's (1957) modification of standard equipment. The subject fixated a small dim red light, and observed the flash of a 1° circular patch situated at 12° from the fovea, continually flashing on for $\frac{1}{2}$ sec, off for $\frac{1}{2}$ sec. The log. brightness was controlled by a photometric wedge driven by a motor, and records were traced upon a rotating drum. The direction of the motor was reversed by a switch operated by the subject in such a manner that when the flash was seen, the motor made it progressively dimmer until finally it was not seen, whereupon the subject reversed the switch and waited until he perceived the flash again. A saw-tooth tracing was thus obtained which continually oscillated about the true threshold.

Now in dark-adaptation measurements the moment of transition from first to second branch is usually detected by the kink in the curves. This kink is apt to show very clearly in the curves which authors draw through their points, but one sometimes feels doubts

when faced with one's own experimental results. Which irregularities are erroneous and should be smoothed out; which is the transition point, which perhaps could be emphasized a little? The use of test flashes with alternating colours has permitted us to locate the transition point securely.

In front of the test flash was interposed either a yellow interference filter (λ = 580 mμ) or a green one (520 mμ) with a neutral density 0·68 fixed to it, making the combination indistinguishable from the yellow in scotopic vision. In photopic vision, however, the yellow threshold was more than half a log. unit lower. These two filters were alternately interposed, being changed every minute throughout dark-adaptation. The result is as shown in Fig. 1. Throughout the first branch of the curve the threshold changed with each change in filter, and the tracing resembles a battlement. Upon the second branch *per contra* the two lights are equivalent and the battlements vanish. The moment of transition could be appreciated by the subject as the point when an exchange of filters suddenly produced practically no change in the colour or the brightness of the test flash.

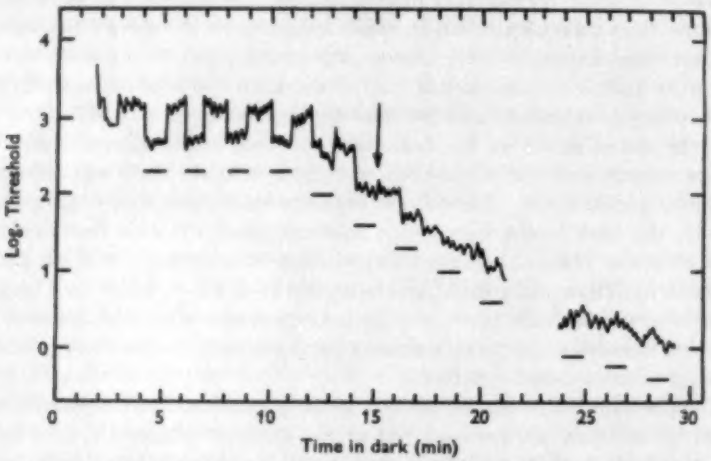

Fig. 1. Dark-adaptation following total bleach. Irregular line is the saw-tooth tracing recorded (with pause at 21–24 min). Test flashes were yellow during the 1 min periods indicated by the short horizontal lines. During the alternate minutes they were green of equal scotopic value. Arrow indicates cone–rod transition for yellow test light.

Measurement of rhodopsin. The retinal densitometer had the simple form indicated in Fig. 2. The light source A was a vertical coiled filament of a 6V car headlamp. From it two beams diverged and were united at the polarizing beam-splitter B, upon which the filament was imaged by lenses L_1, L_2 and the two images were adjusted to coincide. Polaroids P_1, P_2 completed the orthogonal polarization of the two united beams, which were finally reflected into the eye by the tiny mirror M. The lens L_4 focused the filament upon the cornea and at the same time brought sharply into focus upon the retina the image I_1 of the stop S_1, which was uniformly illuminated by coinciding with the image of lenses L_1, L_2 formed by L_3. The light reflected from the eye fell upon L_3 and an image of I_1 was formed at I_2 in the plane of the iris stop S_2, which was shut down so as to be entirely filled by I_2. The light passing through S_2 (and that only) fell upon the cathode of an E.M.I. photomultiplier tube $P.C.$, the output of which was measured.

Principle of measurement. The deep red filter F_1 transmitted a light which was not appreciably absorbed by rhodopsin, so the reflexion of this light into *P.C.* was unaffected by rhodopsin density and hence could be used as a reference standard. The filter F_2 transmitted light of wave-length 516 mμ, which is strongly absorbed by rhodopsin; thus the amount of this light reflected into *P.C.* was less the greater the density of the rhodopsin through which it had twice passed. The polaroid *P* was mounted in a ball race and rotated at a rate of 40/sec. As it rotated it cut off alternately the red and the green light. Thus, if the rotation rate were n radians/sec, the instantaneous output of the photocell would be

$$r\sin^2 nt + g\cos^2 nt,$$

where r and g are the maximal outputs from red and green lights alone and t is time (sec).

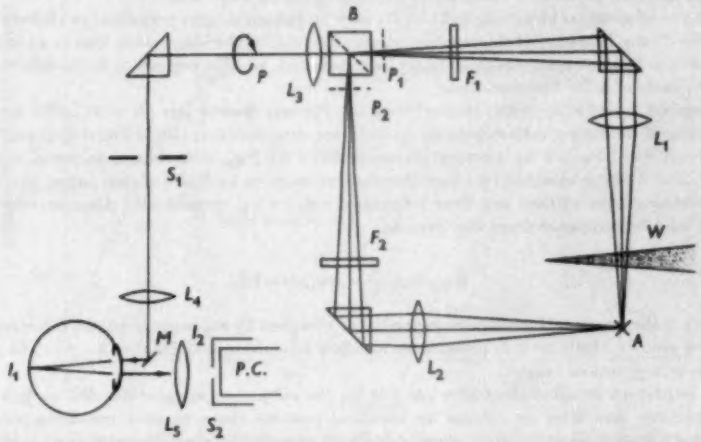

Fig. 2. Diagram of simple form of retinal densitometer. For details see text.

By shifting the photometric wedge *W*, the value of r could be made equal to g, whereupon the output becomes

$$g\,(\sin^2 nt + \cos^2 nt) \equiv g$$

so the a.c. output becomes zero.

It will now be plain how rhodopsin can be measured. The a.c. output is detected and adjusted to zero by shifting *W*. Any increase in rhodopsin density will affect g but not r, and so the a.c. output will no longer be zero. When balance has again been restored the wedge *W* must have introduced into the red path a density increment equal to that introduced by rhodopsin into the green path.

The signal/noise ratio is very much improved by the use of a phase-sensitive rectifier. An additional steady plane-polarized light (not shown in Fig. 2) is passed through the rotating polaroid *P* and focused upon a second photomultiplier cell whose output is *multiplied* by the output from the eye. The integrated product is passed through a sensitive galvanometer which is brought to zero deflexion by suitable setting of the wedge *W*. Now when t is great

$$\frac{2}{t}\int_0^t \sin nt \sin pt\, dt \to 0,$$

except when $p = n$, when the expression becomes unity. In this way the integrals of all noisy components in the signal average out to zero except such as happen to coincide in frequency with n, the signal itself, and are more or less in phase.

Procedure

The subject's pupil was dilated with cyclogyl (cyclopentolate HCl 1·0%: Merck Index) and he was aligned in the densitometer, being kept in position by biting upon a dental impression and steadying his head with a moulded forehead rest. He fixated upon a small red light (not shown in Fig. 2) so placed that the 3° region of retina measured for rhodopsin coincided with the 1° test flash detected during dark-adaptation. This adjustment was secured by removing the filters F_1, F_2 (Fig. 2) so that a bright white light fell upon the retina while the red light was being fixated. Then immediately the subject moved to the dark-adaptation apparatus and adjusted the fixation light there so that the test flash fell upon the after-image still prominent from the white densitometer exposure.

The pre-adaptation bleaching light was a very bright white light presented in Maxwellian view for 30 sec. The subject fixated just outside the edge of the Maxwellian lens at a marked spot, so the fixation spot was not greatly light-adapted, and the regions to be measured lay at the centre of a 20° bleached area.

Campbell & Rushton (1955) showed that the Bunsen–Roscoe law ($It = k$) holds for the bleaching of rhodopsin with exposures up to 45 sec, and Rushton (1956) found that half the rhodopsin was bleached by a retinal illumination of 6·8 $\log_{10}$ troland sec, delivered within 45 sec. The fraction bleached by other illuminations is given by Fig. 6 of that paper, in which the bleaching light without any filter interposed was 7·8 log. troland sec. Alternatively the result may be computed from the formula

$$\log_{10} \log_{10} \frac{1}{p} = \log_{10} It - 7{\cdot}3, \qquad (1)$$

where p is the fraction of rhodopsin remaining unbleached by exposure to a light It (scotopic) troland sec. In this way it is possible to bleach a predetermined fraction $1-p$ of the rhodopsin in any retinal region.

An important detail in the technique was for the subject to acquire the skill to quit the densitometer and later to resume an identical position there, so that measurements of rhodopsin (in equilibrium) were repeatable. With this skill it was not necessary to remain clamped and immovable throughout the long time course of rhodopsin regeneration, and moreover measurements of dark-adaptation could be interpolated.

The actual course of an experimental run was as follows. After a preliminary bleach the subject remained half an hour in the dark, sometimes tracing a curve such as Fig. 1. Then one or two preliminary measurements were made of the wedge settings for the fully regenerated rhodopsin density. The eye was then exposed for 30 sec to the Maxwellian bleaching light at a predetermined intensity level. The subject immediately resumed position in the densitometer and readings were taken of wedge settings, frequently at first, less so as recovery slowed, with results such as those shown in Fig. 3. When the intervals became as long as 2 min the subject left the equipment and rested in the dark between readings. The eyes were always kept shut except during the 7 sec or so during which wedge settings were being made.

For the first run the rhodopsin was bleached completely, so that the change in wedge setting which corresponded to full bleaching was known from the outset. The setting corresponding to 85% regeneration was therefore easily computed. When in each run regeneration was seen to reach this point, the subject left the densitometer and took up position at the dark-adaptation equipment, and at once began to record his threshold, employing the alternate yellow and green flashes matched for scotopic equality.

Without exception, at the time when regeneration was 85% complete, the yellow flash appeared much brighter than the green, showing that cones were still the more sensitive system. Within 5 min, however, conditions changed, and the two lights appeared identical both in brightness and colour. The subject could signal the moment of this change and it

corresponded to the kink in the recorded curve, and to the change from castellation to smoothness. Dark-adaptation measurements were continued for 2 or 3 min to establish the rod curve more securely, and then the subject returned to the densitometer to complete the regeneration curve. In conclusion, the fully dark-adapted threshold was recorded upon the tracing.

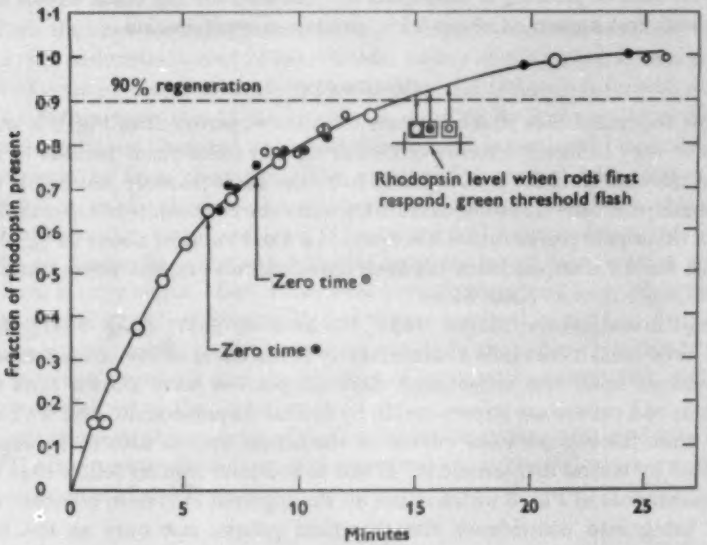

Fig. 3. Regeneration of rhodopsin in the dark. The curve is an exponential of time constant 6 min. All black circles displaced to the right so that zero lies at point indicated. Small white circles similarly displaced but further. For each run the cone–rod transition of the dark-adaptation curve lay where shown by the arrow. ○ 25%; ● 35% bleach; ○ total bleach.

RESULTS

The results of one experiment are shown in Fig. 3, where the large white circles show the fraction of rhodopsin regenerated at various times after a total bleach. The curve is an exponential of half-decay time 4 min. The moment in dark-adaptation when the rods first responded to the green flash is shown by the arrow and corresponds to a regeneration of 92% rhodopsin after an interval of 15 min in the dark.

The black circles represent a repetition of the experiment with a weaker pre-adapting light which bleached 35% of the rhodopsin. These results have been plotted in Fig. 3, displaced 6 min to the right so that zero time falls at the point shown. This is the displacement required to bring the first point into coincidence with the exponential curve, and it is seen that all the subsequent points lie also upon the same curve. The moment when

rods first responded to the green flash is shown by the second arrow, and though this is now only 10 min from zero time it still corresponds to about 92 % rhodopsin regeneration.

The small white circles represent a third run, now with a 25 % bleach. The method of plotting is analogous to that used for the black circles and rods still first appear at about 92 % rhodopsin regeneration.

DISCUSSION

The important fact which appears from the experiment of Fig. 3 is that, despite very different extents of bleaching and subsequent periods in the dark, the threshold for rods is found to be the same (namely just below the dark-adapted cone threshold and 1·9 log units above absolute rod threshold), when rhodopsin regeneration has reached a fixed value of about 92 %. Nor is this fixed correspondence between threshold and regeneration likely to be confined to that point alone.

Most investigators (Müller, 1931; Hecht *et al.* 1937; Haig, 1941; Mote *et al.* 1950) who have made a careful study of the effect of various *substantial* bleachings upon the subsequent dark-adaptation have shown that the various rod curves are superposable by lateral displacement. And we have seen that the regeneration curves of rhodopsin appear also to be superposable by lateral displacement. If this is accepted it must follow that the displacements of Fig. 3 which cause all the pigment curves to coincide will also bring into coincidence the threshold points, not only at the first moment of their appearance which we have tested but throughout.

It must be admitted, however, that measurements of the final 8 % of rhodopsin regeneration are not accurate enough to establish the exact superposability of this tail end of the curves. Our confidence in it rests upon the following argument.

We have seen from Fig. 3 that the over-all regeneration of rhodopsin follows pretty accurately the relation

$$y = y_0 e^{-t/8}, \tag{2}$$

where y is the fraction of opsin still uncombined at time t (min), and y_0 is y at zero time. But from the chemistry of rhodopsin regeneration (Wald & Brown, 1956) we know that

$$\begin{array}{ccc} \text{retinene} + \text{opsin} & \rightarrow & \text{rhodopsin} \\ x \qquad\quad y & & (1-y). \end{array}$$

Thus if x is the concentration of retinene, the rate of regeneration of rhodopsin is given by

$$-\frac{dy}{dt} = kxy,$$

where k is the velocity constant. But differentiating (2) gives

$$-\frac{dy}{dt} = \frac{1}{6}y_0 e^{-t/6} = y/6.$$

$$\therefore \quad x = 1/6k \tag{3}$$

Thus the exponential regeneration of rhodopsin implies a constant value for x, the concentration of 11-*cis* retinene, as has already been shown in the case of cone pigments (Rushton, 1958). Now the retinene is formed either by oxidation from the 11-*cis* vitamin A store or by isomerization of the all-*trans* retinene liberated upon bleaching, and it is removed by combining with opsin to form rhodopsin. The exponential curves show that when removal is relatively fast the retinene replacement maintains the concentration x at a very fixed level. Thus it would be surprising if this level could no longer be maintained in the later stages of regeneration when removal is only slight. But if the level here does remain fixed, the curves must remain strictly exponential, and we are justified in our belief in their precise superposability. From this we conclude that there is a fixed relation between threshold and the fraction of opsin still uncombined, and we may now ask what that relation is.

Each member of the series of dark-adaptation curves obtained by Haig (1941) after various bleaching exposures was shown to fall upon a mathematical curve of the form

$$\log I/I_D = ae^{-t/b}, \tag{4}$$

where I_D is the dark-adapted threshold and a, b are constants. It is true that the equation quoted by Haig looks much more complex than (4) but it may be reduced to this form by a suitable change of constants. Haig found the time constant b to increase with increased bleaching, but for bleaches greater than 25 %, b reached an upper limit of about 6 min. Now 6 min is the time constant for rhodopsin regeneration, so combining equations (4) and (2) we obtain

$$\log I/I_D = ae^{-t/6} = ay/y_0. \tag{5}$$

Thus, if threshold is expressed in units of the dark-adapted value I_D, we derive the required relation—log. threshold is proportional to the fraction of opsin still uncombined. This is the relation which Wald, Brown & Smith (1955) have deduced (for small bleaches) from the 'compartment hypothesis' of Wald (1954). It receives experimental support from the impressive paper of Dowling & Wald (1960) which has just appeared, wherein several important changes in structure and function of the rat's retina are studied in relation to the removal of rhodopsin. One aspect of this work concerns the change in log. threshold which accompanies the bleaching

and regeneration of rhodopsin. Albino rats had all their rhodopsin bleached away by exposure to bright light. Then, during the subsequent stay in the dark, the e.r.g. was determined at different times in response to flashes of various strengths. The rhodopsin present at each state was found by killing a rat and extracting its rhodopsin, one pair of retinas yielding a solution from which a measurement could be made with good precision. Experimental details are not given, but this exacting work appears to have been well performed, and demonstrates good linearity between the rhodopsin content and the log. threshold for the first appearance of the e.r.g. This investigation therefore established experimentally for the rat the linear log. threshold relation which we confirm in man at a very different level of visual activity.

Dowling & Wald (1960) then went further and extended the relation to the condition of night blindness which follows deprivation of vitamin A from the diet. Young rats were reared upon food lacking vitamin A but containing vitamin A acid. They grew well and appeared normal in health except for the rods, which at first lost rhodopsin and later lost their structure and degenerated. During the period of 7–10 weeks, throughout which rhodopsin progressively fails but structural change is not yet severe, the e.r.g. at maximum is nearly normal in size, though it needs a much stronger flash to elicit it. The relation between the e.r.g. log. 'threshold' and the fraction of rhodopsin present, as compared to normal, at each stage is again linear. And the line is the same one which fitted the relation described above. Thus removal of rhodopsin either by bleaching or by vitamin lack raises the 'threshold' in identical fashion.

Now the 'threshold' for an e.r.g. is some 1000 times higher than that for rod vision, so one should hesitate to jump from relations about e.r.g. in the rat to perceptual thresholds in man, though Karpe & Tansley's (1948) work goes some way to support this. The present results, however, do show rather directly that the same logarithmic relation applies.

In this paper the evidence that log. threshold depends immediately upon rhodopsin content is fairly straightforward, but the grounds for believing the relation to be linear are more indirect. We are strengthened in this belief, however, not only from Dowling & Wald's results upon the rat, but also from some observations upon a 'rod monochromat' where the same relation could be demonstrated upon a far more extended scale. This is described in the papers immediately following.

SUMMARY

1. Dark-adaptation curves were traced by using a test flash which alternated in colour each minute between yellow and green. The coloured filters were adjusted for scotopic equality, thus they were indistinguishable on the rod branch of the curve (Fig. 1) but exhibited a battlement tracing upon the cone branch. In this way one knew at each minute whether cones or rods were involved.

2. The regeneration of rhodopsin was measured by reflexion densitometry in the normal eye following light adaptations which bleached various fractions of rhodopsin from 25 to 100%. Regeneration always returned along an exponential curve of 4 min half-return time.

3. During each run of pigment measurement the procedure was interrupted for a few minutes to permit some dark-adaptation measurements to be made. These always included the cone–rod transition, which occurred at about 2 log. units above the full dark threshold.

4. At the moment of the transition the rhodopsin had always regenerated about 90% independently of the amount bleached initially.

I should like to thank Dr R. D. Gunkel for the loan of his dark-adaptation equipment and for valuable assistance in the course of these experiments, and I am grateful also to all those who offered themselves as subjects in this work. Above all am I indebted to Dr M. G. F. Fuortes, who proved to be the most steady and consistent subject I have ever had. From his eye are taken the records shown in this paper: to his skill is due such accuracy as has been achieved.

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INCREMENT THRESHOLDS IN A SUBJECT DEFICIENT IN CONE VISION

BY M. G. F. FUORTES,* R. D. GUNKEL AND W. A. H. RUSHTON†
From the Ophthalmology Branch, National Institutes of Health, Bethesda, Maryland, U.S.A.

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It is well recognized, particularly from the work of Hecht (1937), that measurements of visual function at different levels of illumination generally fall into two categories, a low intensity scotopic portion and a high intensity photopic portion. Thus in the time course of dark-adaptation, and in the curves where log. increment threshold, log. acuity or flicker fusion frequency is plotted against log. field intensity, the results are found to fall upon two distinct branches. The low-intensity branch has the spectral sensitivity of rhodopsin, and it is not seen in measurements made upon the fovea centralis; it is therefore considered to be due to rod function. The other branch has a different spectral sensitivity, it is found upon the rod-free fovea and hence it is considered to be due to cone function.

Now though there are considerable difficulties in distinguishing the functions of the various types of cone, it is easy to separate cone curves from rod curves over the whole intensity range of the cones. For not only may measurements be made upon the fovea where rods are absent, but even elsewhere it turns out that cones have an increment sensitivity and fusion frequency so much higher than rods that the plotted curves generally show the cone branch substantially in its entirety.

It is otherwise with rod curves, for not only is there no part of the human retina which contains rods without cones, but the very features which make cone measurements outstanding in a mixed population make rod measurements unavailable. In fact, we can only measure rods at intensities below the cone threshold. This does not mean that rods are necessarily inactive at higher intensities, but their activity cannot be measured by ordinary threshold procedures, and we are left wondering what course the rod branch may take after it disappears behind the cone branch.

This question could be answered if we were able to investigate a subject who had no cones but whose rods and rod pathways to the brain were

* Instituto Neurologico, Via Celoria 11, Milano.
† Present address: Physiological Laboratory, University of Cambridge.

normal. The subject to be described in this paper and in the sequel comes near enough to that condition to be of interest.

Photanopia. A rare congenital condition resembling pure rod vision has been recognized for about a century and has played an important part in establishing the duplicity theory of vision (see the historical review in Walls & Heath, 1954). The subjects have little or no colour vision and are hence called 'achromats' or 'monochromats', more specifically rod-monochromats, to distinguish them from an entirely different type of achromat with good photopic vision (cone monochromats). But all the subjects fully investigated have shown features inconsistent with what would be expected of an eye with normal rod vision and nothing else. They have generally exhibited some features resembling a limited cone vision (Lewis & Mandelbaum, 1943; Hecht, Schlaer, Smith, Haig & Peskin, 1948). The degree and the manner in which cones enter varies considerably in reported cases and the cone spectral sensitivity may be maximal either at 440 mμ (Blackwell & Blackwell, 1959), 510 mμ (Sloan, 1954, 1958; Alpern, Falls & Lee, 1960) or in our case at 550 mμ. There is no reason to believe that all these represent the same genetic condition, and indeed the mode of inheritance of the 'blue cone' type appears to be different from that of the 'rhodopsin cone' type (H. F. Falls, personal communication). It seems convenient to have a name shorter than 'incomplete typical achromatopsia' to describe these various cases of very deficient photopic vision, and we shall use 'photanopia' to include them all.

Our object here is to study rod vision in one photanope for the information this may give concerning normal rod vision, and so we shall not be concerned except incidentally with the complex and fascinating questions as to the nature of the cone malfunction. This matter has been discussed extensively by Walls & Heath (1954) and investigated by Alpern *et al.* (1960) with a very impressive range of techniques.

Description of the photanope D.M. She was 17 years of age with no brothers and one younger sister who was said to exhibit the same condition in milder form. There was no known comparable visual defect in parents or relations. D.M. had had the condition all her life and some years back exhibited a marked nystagmus which has now largely vanished (as it usually does in this condition with advancing years).

She saw well (i.e. normally) in dim light, but with increasing illumination she saw worse, and upon questioning it appeared that contrast was lost and the whole field assumed a bright undifferentiated appearance. A retinal illumination of 1000 td would produce this effect, and produce it instantly, and upon return to 1 td the sense of contrast returned within a second or so. It therefore was not linked with the bleaching and regeneration of rhodopsin which takes 4–5 min to recover half way (Rushton, 1961).

Although she did not like bright illumination, it gave her no pain, and she could submit to 30 sec fixation upon 1,000,000 td of retinal illumination without complaint and without the subsequent 2 hr of disability described by Walls & Heath (1954).

Visual acuity was 20/80 as measured by the Snellen chart (no serious error of refraction). Colour vision was nearly absent, but good red or blue lights were always correctly named. Blue-green and yellow were both called grey, and she failed in all ordinary colour-vision tests.

It is important but not easy to know whether the fixation point falls upon the fovea or not. When an ophthalmoscopic arrangement focused a bright 2° field upon the retina and D.M. was asked to fixate the centre of it, the illuminated retinal region was seen to have a point of light (foveal reflex) situated near the centre. This point of light is the real image formed by the ingoing beam after reflexion in the foveal cup. The point is situated about 30 μ in front of the centre of the cup. Thus if the fovea is defined as the region of the cup the illuminated retinal image was seen to be situated near the centre of the fovea, and hence in these conditions D.M. fixated with her fovea. However, this is not to say that she did so in other conditions of illumination.

D.M. was a very favourable subject for this work because her rods appeared quite normal and such cones as she possessed had familiar properties and hence could easily be understood. Moreover she was most co-operative, with quick understanding, sharp observation and sustained attention. Nevertheless, in spite of long hours and repeated sessions much of the work is less complete and well confirmed than could be wished, but the period at our disposal was limited.

In this paper we investigate the increment threshold, and in the sequel the course of dark-adaptation in relation to the regeneration of rhodopsin.

METHODS

The optical arrangement of Fig. 1 was designed to superpose a small flashing light upon a background field and to present these to the subject by Maxwellian view, in such a manner that the points of entry of flash and field through the pupil could be adjusted to centre or periphery. The light source was a zirconium arc (not shown) whose crater was focused by an achromatic lens upon the stop S_1 of 3 mm diameter. The light could be attenuated by neutral filters F_c in the common path. Light entering through S_1 was divided by the beam-splitter B_1, and each beam could be modified by neutral wedges W_1, W_2 and neutral and narrow-band interference filters F_1, F_2. The beams were recombined at the beam-splitter B_2 and fell upon L the Maxwellian lens, which imaged S_1 upon the pupil at $\frac{1}{4}$ of the size. Beam 1 usually filled the lens and provided the background field; beam 2 was restricted by stop S_2 and subtended a 1° patch in the centre of the lens (see inset, Fig. 1). The rotating sector R continually interrupted the light so that the 1° patch was on for half a second and off for half a second. A small fixation light D was placed at the edge of the lens L when parafoveal increment thresholds were measured. The brightness was adjustable and the distance from the 1° patch was 7°.

It was sometimes required to send the flashing light through the centre of the dilated pupil (8 mm diam.) and the background field through the periphery, or vice versa. The separation of the two focal points of entry upon the pupil as indicated by the inset of Fig. 2 was adjusted by rotating B_3 in the horizontal plane. The shift across the pupil was achieved by a horizontal movement of L across the beam. Mechanical stops were fixed so that the lens could instantly be pushed from one position to the other to change between situations A and B (inset, Fig. 2). When the light is passing through the pupil, it is not easy to see the point of entry; that only becomes obvious when the light is caught upon the iris muscle.

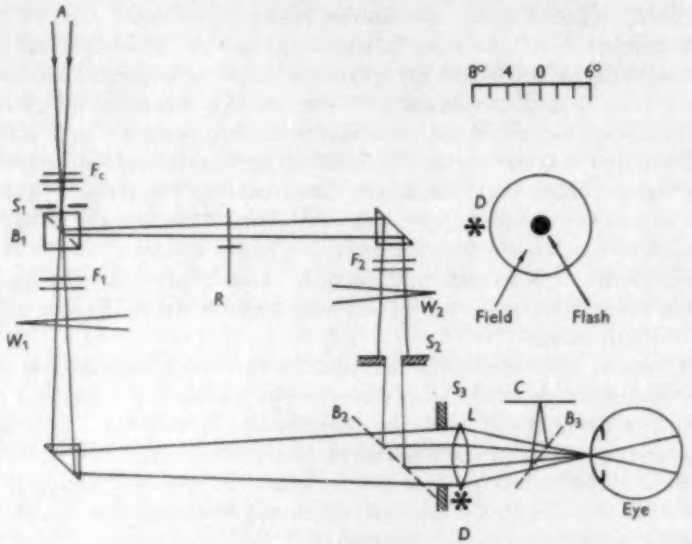

Fig. 1. Apparatus for measuring increment thresholds. Inset: Visual field. A, light from zirconium arc focused upon stop; B_1, B_2, B_3, beam-splitters; C, white card; D, fixation light; F_1, F_2, interference filters; F_c, neutral filter; R, sector disk, $\frac{1}{2}$ sec on, $\frac{1}{2}$ sec off; S_1, S_2, S_3, stops; W_1, W_2, wedges.

The beam-splitter B_3 (Fig. 1) was found very helpful, for by placing the white card C to coincide with the image of the pupil, the two points of entry upon the pupil also appeared as bright points upon the card. Then, looking through the beam-splitter, these points could easily be seen, and so could the whole reflected eye, if a weak light was shone upon the subject's face. In this way the two focal points could be seen clearly in relation to the pupil as in the insets A, B (Fig. 2) and the entry points properly adjusted. B_3 was left in position to check from time to time the possibility of small head movements which could impair the setting.

The subject bit upon a dental impression, her head was steadied by a forehead rest, and she fixated either the central flashing 1° field or else the red fixation light D, 7° away. The pupil was dilated with cyclopentolate HCl 1%.

Calibration of interference filters. The interference filters were all about 10 mμ band-pass at half maximum. They were treated as though they transmitted monochromatic light at peak wave-length. We needed to know the relative energies of the light incident upon the cornea. An E.M.I. red-sensitive photomultiplier cell was covered except for a 2 mm hole

drilled in a metal cap above the photocathode. The cell was substituted for the subject's eye so that the $\frac{1}{2}$ mm focal point of light entered the 2 mm hole and the light fell upon the cathode. An opaque screen was interposed at F_1 and the wedge W_2 (Fig. 1) was adjusted so that the output of the photocell was always the same whatever the interference filter F_2. The spectral sensitivity of the photocell was known and thus the relative quantal flux could be directly calculated. The calibration was made in beam 2 (Fig. 1) and all the spectral sensitivity measurements were made in this beam; thus the calibration gives directly the relative quantum flux at the cornea.

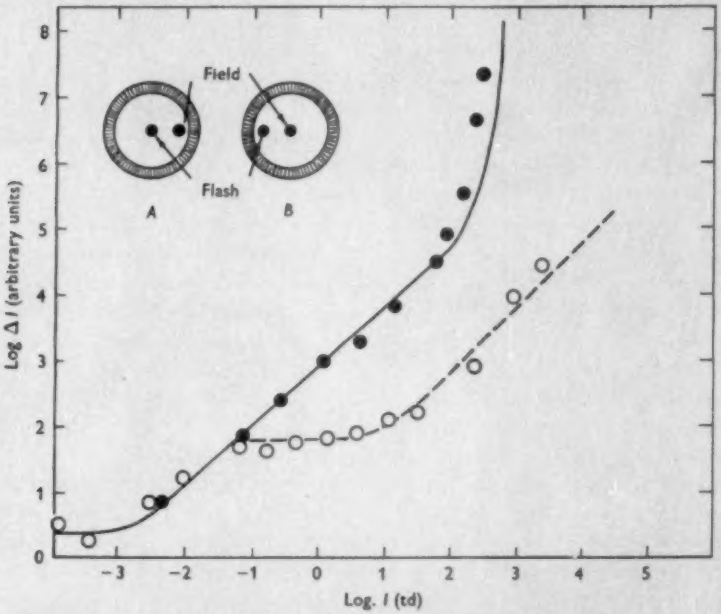

Fig. 2. Increment threshold 7° parafoveal. Ordinate, log. increment flash intensity (arbitrary units); abscissa, log. background field. ○, normal subject; ●, photanope; continuous curve, normal rod increment threshold curve (from Aguilar & Stiles, 1954). Inset: Points of entry of flash and field lights through dilated pupils in two cases *A* and *B*.

RESULTS

Parafovea

In Fig. 2 are plotted some increment thresholds with the adaptation field in log. trolands as abscissa and the superposed threshold flash as ordinate also upon a logarithmic scale. The white circles show the result upon a *normal* subject with our apparatus obtained with yellow light (580 mμ) for the flash, fixation 7° temporal to the test-flash and with both lights sent through the centre of the pupil. The curve is familiar from the work of Stiles (1939, 1949, 1953). It consists of two branches each of the

same characteristic shape and each corresponding to one visual mechanism. In shape each branch is a horizontal line which runs indefinitely from the left (the absolute threshold for the mechanism) and turns rather sharply to become a line of slope nearly 45° (the Weber–Fechner relation). The lower branch to the left corresponds to rod vision. At a field strength of -1 log. td the cone threshold is reached, and a second branch of the curve is obtained corresponding to one or more of the cone mechanisms, as Stiles has demonstrated very fully.

Now if the photanope has normal rods and no cones in the retina at a point 7° temporal to the fixation spot, we should expect the above experiment to give a normal lower branch to the curve but no upper branch. The black circles (Fig. 2) show that this expectation is exactly fulfilled, and the Weber–Fechner line continues to rise for 3 log. units above the point where it is normally hidden by the greater sensitivity of the cones. At a background field luminance of about 2 log. td, however, the rod curve begins to change, and in a sense contrary to that expected from the entry of a new mechanism. For instead of thresholds appearing below the Weber–Fechner line, at about 2 log. td they rise above it, and at 3 log. td of background illumination the brightest white flash we delivered remained undetected.

Now though in normal subjects at moderate illumination rod function is usually masked by cone activity, Aguilar & Stiles (1954) have succeeded in demonstrating it by an ingenious technique. The activity of cones was depressed by using as background field a red light introduced through the centre of the pupil, and the test flash excited the rods relatively well by being green and by entering through the periphery of the pupil. With this technique the threshold of the cones was so far raised that the flash only began to excite them at a background field of 3 log. td or more. It was therefore possible to plot the increment threshold curve for rods in normal subjects up to a background field of 3 log. td. Their results are shown as the unbroken curve in Fig. 2 and it is seen that our results upon the photanope agree with theirs upon the normal subject so well that the two curves may nearly be superimposed.

Test upon the fixation point

When the background field was 3 log. td of retinal illumination our rod monochromat could not see the brightest flashes when she fixated 7° away from the 1° flashing light. But when she looked directly at the flashes she could see them. Thus she clearly possesses at the fixation point a visual mechanism that does not become ineffective at a retinal illumination which incapacitates normal rods. Figure 3 shows an experiment, plotted as Fig. 2, which throws some light upon this mechanism.

At each background intensity the photanope made two increment threshold determinations (each repeated) one fixating upon the test flash and one upon the fixation light (*D*, Fig. 1) 7° temporal. Both field and flash entered the dilated pupil coincident and near the centre in both fixation positions. The flashing beam passed through a 580 mμ interference filter, the field through a 520 mμ filter together with a 0·68 neutral, which gave it the same scotopic brightness as the 580 mμ filter alone.

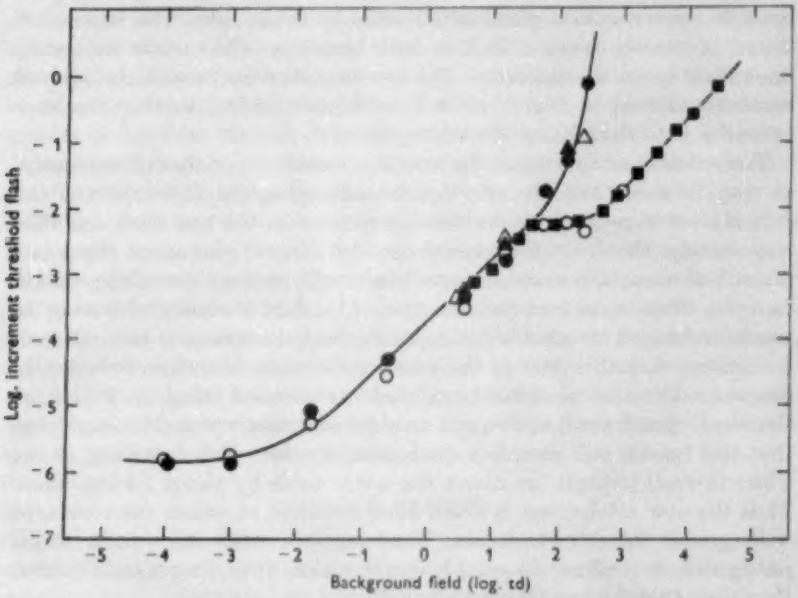

Fig. 3. Increment thresholds of photanope with flash and background of wave-lengths indicated below.

	Wave-length (mμ)	
	Flash	Background
○ fovea	580	510
● parafovea	580	510
△ fovea	510	580
▲ parafovea	510	580
■ fovea	White	White

The results of Fig. 3 were taken in the order from left to right. The first point had zero background field, so this measures the absolute threshold, but the subject had not remained in the dark long enough for it to represent the fully dark-adapted threshold. It is seen that for all backgrounds from zero to +2 log. td the threshold is the same when fixation was central

(white circles) or peripheral (black circles), and the results correspond well enough to those of Fig. 2. But at backgrounds higher than 2 log. td there is a marked divergence in the curves. With peripheral fixation the threshold rapidly rises above the Weber–Fechner line and exhibits the features of normal rod saturation seen in Fig. 2. With central fixation, on the contrary, the threshold falls below the line and evidently indicates the appearance of a new visual mechanism. In order to investigate this mechanism more extensively the colour filters were removed from flash and field and a two-branched curve was obtained as indicated by the squares. The removal of filters, of course, causes a shift in both branches of the curve depending upon their spectral sensitivity. The two-branched curve with the squares has been plotted in Fig. 3, shifted (without rotation) so that the kink coincides with the kink of the white circles.

The spectral sensitivity of the new mechanism is not that of rhodopsin, as may be seen from the effect of interchanging the (580 mμ) and the (520 mμ + 0·68 neutral) filters which had been in the test flash and field respectively. Both for the normal eye and for our photanope these two filters had about the same scotopic brightness, so their interchange could have no effect upon pure rod function. At a field intensity of 0·5 log. td the interchanged thresholds (triangles for circles) are seen to be unaltered, confirming that this part of the curve records the activity of rhodopsin receptors. The next point to the right shows the same thing. At 2·2 log. td the black (parafoveal) circles and triangle still nearly coincide, signifying that this branch still records a rhodopsin receptor. But at 2·4 log. td the white (foveal) triangle lies above the white circle by about 1·4 log. units. Thus the new mechanism is much more sensitive to yellow (as compared with green) than is rhodopsin. This suggests that the second visual mechanism at the fixation point is due to cones. If so, they should exhibit the Stiles–Crawford retinal direction effect.

Stiles–Crawford effect

As is well known, Stiles & Crawford (1933) showed that light entering through the centre of the pupil is much more effective in stimulating cones than is light which enters through the periphery of the dilated pupil and falls upon those same cones. But this retinal direction effect applies to cones only, and hardly or not at all to rods (Stiles, 1939; Flamant & Stiles, 1948). Thus if the flash and background beams enter the pupil in different places, as in Fig. 2 (inset), it should not make any difference to the *rod* threshold whether light entered the pupil as at A or at B. But if cones are excited in a situation where the increment threshold lies upon the Weber–Fechner 45° line, a change from A to B should increase the log. increment threshold by twice the change expected

from moving the entry point of either flash or field alone, i.e. we might expect a total change of about 1 log. unit.

In the experiment of Fig. 3 a change of pupil entry from A to B caused an increase in threshold of 0·1 log. unit in the lower region of the curve, but it was otherwise with the second visual mechanism. With conditions corresponding to the last circle to the right (Fig. 3) the shift from A to B caused 0·5 log. unit of threshold change.

Alpern *et al.* (1960) have measured the Stiles–Crawford effect in some photanopes more thoroughly than we did. Using a flicker method they plotted the efficacy of the light as a function of its point of entry through the pupil and found a marked asymmetry, indicating tilting of the foveal cones. The magnitude of the Stiles–Crawford effect therefore appeared greater or less than normal, depending upon the particular point of entry round the edge of the pupil, and our low value may well have been due to the one peripheral point we used chancing to be at a low-valued region.

We conclude that the receptors responsible for the main curve of Fig. 3 are normal rods, since they have the spectral sensitivity of rhodopsin, the absence of Stiles–Crawford effect and they coincide with Aguilar & Stiles's (1954) curve for normal rods, becoming saturated at the normal value of 3 log. td. The receptors of the high-intensity branch, on the other hand, appear to be cones, for they are more yellow sensitive than is rhodopsin, and they exhibit the Stiles–Crawford effect. But they differ from normal cones in that they are only detectable on or near the fovea, their threshold is very high, and their acuity is poor.

Spectral sensitivity

From the results of Fig. 3 it was hardly to be doubted that the spectral sensitivity measured at absolute threshold would be that of rhodopsin, whether at 7° parafoveal or by direct fixation. We therefore contented ourselves with the rather irregular results of Fig. 4 (black circles and triangles) which roughly correspond to the rhodopsin sensitivity shown by the continuous curve (that plots the log. absorption of rhodopsin against wave number).

Of more interest is the spectral sensitivity of the upper branch of the curve in Fig. 3, namely the mechanism left when a bright background field has abolished the response of rods to a superimposed flash.

The intensity of the white background was raised until no increment flash was detectable at 7° parafoveal even with a strong white flash. Now the subject fixated directly and was able to see the flash even when greatly reduced. By interposing a filter transmitting either 492, 527, or 590 mμ the threshold energies for these lights could be found. The white circles of Fig. 4 show the average quantum energies obtained plotted as log.

sensitivity. The interrupted curve is the sensitivity of a visual pigment which fits Dartnall's nomogram (1952) and has its maximum at 550 mμ. This shift in spectral sensitivity corresponds closely to the change seen in Fig. 3 when the green and yellow filters in flash and background were reversed.

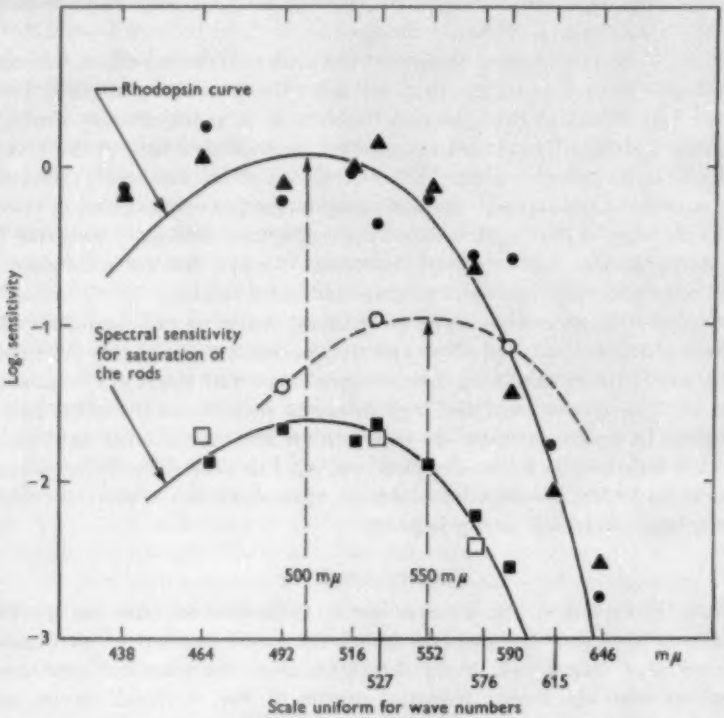

Fig. 4. Spectral sensitivity for various visual processes. Ordinate, log. sensitivity (arbitrary units); abscissa, wave-lengths plotted upon a scale uniform for wave frequency. Continuous curves, log. absorption of rhodopsin; interrupted curve, the rhodopsin curve displaced to give maximum at 550 mμ. ▲ parafoveal threshold; ● 'foveal' threshold; ○ foveal increment threshold upon a background which saturates rods.

Now in Fig. 3 it will be noticed that the level at which the rods lose their capacity to signal a superposed flash is just about the level where cones enter the picture, so those who hold that cones can inhibit rods may incline to the view that it is the entry of the cones which causes what we (following Aguilar & Stiles, 1954) have called 'rod saturation'. According to this view, inhibition of the rods will be caused by background lights of

various wave-lengths when the cones have been stimulated to a fixed extent. The spectral sensitivity of a background which just inhibits the rods should therefore correspond more or less to the white circles of Fig. 4, which represent the sensitivity of the cones actually present.

In the experiment to find the spectral sensitivity of the saturating background, the stop S_2 (Fig. 1) and the rotating shutter R were transferred to the other beam, so the subject by moving the wedge W_2 as usual could vary the background field. The test flash was white and was made very bright so that its exclusion would correspond to fairly complete saturation or inhibition of the rods. The black squares (Fig. 4) represent an attempt to investigate many points in the spectrum, the white squares give the results of another experiment where just three wave-lengths were chosen and re-checked several times.

The points are seen to lie close to the (continuous) curve of rhodopsin sensitivity displaced vertically downward, but they cannot be fitted by vertical displacement of the (interrupted) curve of cone sensitivity. Thus the abolition of rod increment function is due to quanta absorbed by rods and not by cones.

DISCUSSION

The photanope whom we studied appeared to have normal rod function, and her rod increment threshold curve on the parafovea coincided with that of Aguilar & Stiles (1954) in their special conditions which excluded increment thresholds of the cones. In particular, rod saturation was reached at the same value of about 1000 td.

When their paper appeared there seemed three kinds of explanation for the abolition of increment perception against this moderate background.

(*a*) Rhodopsin might be all bleached away at this illumination. This was rejected by Aguilar & Stiles in consideration of the small incidence of quanta per second per rod, and more recently the direct rhodopsin measurements of Campbell & Rushton (1955) have confirmed their conclusion.

(*b*) Rods might be inhibited by cones, so that though rods are still active in responding to light they do not send a signal down the optic nerve fibres. This is a plausible concept, for the electrophysiological records from ganglion cells of animals usually show both rods and cones converging upon the same optic nerve fibre, the rod signal preponderating in dim vision, the cone signal in bright (Granit, 1947). It is reasonable to think that the rods have sole use of the common communication line below the cone threshold, but that some mechanism is needed to turn off the rods at high illumination, otherwise all the channels might be jammed full of high frequency rod discharges and be insensitive to the near-threshold cone response which in fact is the signal transmitted.

The results of this paper, however, are very difficult to reconcile with inhibition of rods by cones, for our photanope, though apparently quite devoid of active cones in the parafoveal region, nevertheless, exhibited the 'inhibition' of rods there precisely as does the normal subject, and at the same background of 1000 td. The evidence is even stronger; for the spectral sensitivity curve Fig. 4 (squares) shows that it is rods not cones upon the background field that catch the quanta which abolish increment threshold.

(*c*) There are two ways in which rods might become 'saturated'. (i) The signal generated by each rod in response to light will increase with illumination but only to some fixed upper limit, and when this is reached an increment flash can do nothing further. Upon this view, saturation is a property of the light transducer mechanism of the rod. (ii) Rods may in fact be inhibited not by cones but by other rods. In electrophysiological studies of ganglion discharges from the cat's retina Donner & Willmer (1950) confined themselves to ganglions whose spectral sensitivity was throughout that of rhodopsin. The somewhat bewildering diversity of on/off patterns of discharge which they recorded from various receptors at different intensities makes their conclusion readily acceptable that some interaction between rod and rod may be exhibited in the time course of discharge. A clear case of spatial interaction which varied with the level of dark-adaptation was studied by Barlow, FitzHugh & Kuffler (1957*a*, *b*), in the cat. In their situation both rods and cones generally interacted but there was no evidence of inhibition of rods by cones; rather the rod–cone complex of the centre of a ganglion's receptive field was inhibited at high illumination by the similar rod–cone complex of the periphery.

The electrophysiology of vision is embarrassing in urging us to explain, by nerve discharges of the utmost complexity and variety, visual phenomena which had appeared both uniform and simple. Here we go no deeper than to point out the alternative mechanisms of 'rod saturation'. However, in view of the results of the following paper the first alternative of (*c*) seems the more likely.

CONCLUSIONS

1. In our photanope the rods and rod vision appeared normal, judged from all the experiments we have made as described in this and the following paper.

2. At a background illumination of 1000 trolands the rod increment threshold was abolished in the photanope as it is in the normal subject, and the abolition is not caused by cones. This in fact is the level of illumination at which our subject lost the sense of visual contrast in objects around her and became virtually blind.

3. Cone vision was restricted to the neighbourhood of the fovea. The spectral sensitivity was roughly normal (maximum 550 mμ) and there was a Stiles–Crawford effect. But the cone organization was not normal, since threshold was extremely high, colour vision practically absent and acuity very poor.

SUMMARY

1. These investigations were all made upon one 'incomplete rod-monochromat' (with some comparison upon normal subjects).

2. Increment thresholds measured upon the fovea showed a normal rod branch and also a cone branch with spectral sensitivity maximal at about 550 mμ but with threshold some 2 log. units above that of normal cones.

3. At 7° away from the fovea only rods were active, and the increment threshold relation coincided with the normal rod relation as measured in full extent by Aguilar & Stiles (1954).

4. At a background of about 1000 trolands normal rods become saturated and so do the monochromat's rods, and this is the level where she lost all luminous contrast from her surroundings.

5. The spectral sensitivity of the background which just saturates is that of rhodopsin. Thus the abolition of rod function in monochromat and normal subject by a bright background is not due to inhibition of rods by cones. For it occurs in normal fashion where there are no cones, and rhodopsin is the pigment which catches the quanta.

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RHODOPSIN MEASUREMENT AND DARK-ADAPTATION IN A SUBJECT DEFICIENT IN CONE VISION

By W. A. H. RUSHTON

*From the Ophthalmology Branch, National Institutes of Health, Bethesda, Maryland, U.S.A.**

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This paper is the third in a succession, of which the first (Rushton, 1961) will be referred to as paper I, the second (Fuortes, Gunkel & Rushton, 1961) as paper II. In paper II we described some studies upon a photanope—a subject whose photopic vision was very defective, but whose rod vision appeared to be normal. In particular, the increment threshold, obtained from a retinal region distant 7° from the fovea, coincided with the results of Aguilar & Stiles (1954), obtained from normal eyes when special precautions were taken to exclude the contribution from the cones. Now a subject with normal rods, but practically no cone responses, might be expected to give very valuable information upon the relation between log. sensitivity during the course of dark-adaptation and the regeneration of rhodopsin.

In paper I the course of rhodopsin regeneration was measured in the normal eye, and was found to develop according to a monomolecular reaction with time of half regeneration 4 or 5 min. It was seen, moreover, that after substantial bleaches of various amounts, when rod vision first appeared (at about 2 log. units above the final threshold I_0), rhodopsin was always about 90% regenerated. It was argued that there was a fixed relation between the threshold I and the fraction of opsin still uncombined with retinene—in fact, that this fraction was proportional to log. (I/I_0).

It was impossible, however, to test this relation experimentally over a range greater than 10% of bleaching, since this already causes the rod threshold to rise to the level of that for the cones, and with further bleaching all vision appears to be cone vision and rod thresholds ascend upon a curve of conjecture somewhere in the space above the horizontal cone branch.

But a subject without cones would not in this way conceal the ascending rod curve, and we might hope to follow rod dark-adaptation through a much greater range. This hope has been fulfilled, and we have been able to

* Present address: Physiological Laboratory, University of Cambridge.

plot a rod dark-adaptation curve over a range of nearly 7 log. units and to correlate this with rhodopsin regeneration measured physically upon the same subject.

MEASUREMENT OF VISUAL PIGMENT

Rhodopsin

The apparatus and principles of measurement were those already described in paper I, and the procedure was very similar. The subject, with fully dilated pupil, was aligned in the densitometer and held in position by a dental impression and a moulded forehead rest. After remaining in the dark for 30 min she resumed her position in the densitometer, and wedge settings were taken to give the value corresponding to the fully regenerated condition. Then the subject turned her head and received the bleaching exposure. This was a 20° Maxwellian field of 10^6 td for 30 sec, which should bleach 97 % of the rhodopsin in the retinal area involved (see formula 1, paper I).

The subject tolerated this well, and was seen neither to blink much nor shift her gaze from the fixation point placed just outside the Maxwellian lens. She returned at once to position in the densitometer and fixated upon the small red light (always recognized as 'red') situated 12° from the region measured. This region was a 3° circle near the centre of the 20° area bleached.

The subject remained in position for the next 40 min with eyes closed except for brief periods of about 7 sec during which wedge settings were being taken. At the end of 40 min no further regeneration of pigment occurred, and the initial full dark level had been reached.

Rhodopsin measurements with our photanope were not so self-consistent as with the best of our normal subjects. This may have been due to her difficulty in maintaining accurate fixation, for her central acuity was poor, and there persisted a tiny residue of her earlier nystagmus. The irregularities are greatest within the first few minutes after bleaching, and it is probable that this enormously bright light left a temporary impairment of steady fixation, as indeed for a few seconds it does with normal subjects.

The filled circles of Fig. 1 show one such measurement of rhodopsin regeneration in the photanope; the open circles show it in the normal subject. Both ordinates are expressed as fraction of total rhodopsin present, and the actual amounts of rhodopsin, as measured by change in wedge setting after a full bleach, do not differ by more than 10 % from one to the other.

We may conclude, therefore, that the photanope has about the normal amount of rhodopsin in the retina at 12° temporal to the fovea and that it regenerates at the normal rate.

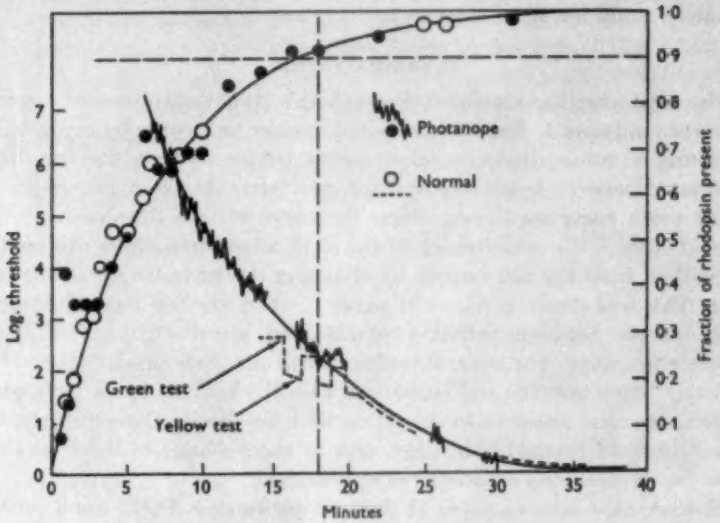

Fig. 1. ○, regeneration of rhodopsin in a normal subject; ●, in the photanope. Irregular curve, dark-adaptation curve of the photanope; interrupted curve, the same experiment with a normal subject.

Foveal pigment

An attempt was made to measure any visual pigment that there might be upon the photanope fovea by means of a 2° measuring field centrally fixated. The bleaching exposure was for 30 sec to a white light of 10^6 td, which should have bleached all the cone pigments present, but measurements with lights of 500, 540 and 580 mμ all failed to detect reliably any change in foveal reflectivity. These central measurements were less steady than the peripheral rhodopsin values, because the macula lutea and foveal reflex emphasize irregularities due to eye movements. Moreover, cone pigments regenerate fast, and thus can only be detected within a minute or two of bleaching, and this is just the period where that intense light leaves irregular fixation.

Despite these uncertainties, an amount of pigment corresponding to 10% of that present upon the normal fovea could perhaps have been detected—20% with some confidence. Neither rhodopsin nor cone pigments were found in this amount, nor indeed was it to be expected, judging by visual function. For our subject had an acuity of 20/80, which might correspond to a coarsening of the mosaic grain of the fovea by $\frac{1}{4}$, linear, or $\frac{1}{16}$ by area. Thus, if all the cones present had normal pigment,

the total pigment change would only be $\frac{1}{16}$ of normal, which in this subject certainly could not have been seen.

DARK-ADAPTATION

The apparatus was Gunkel & Bornschein's (1957) adaptometer, used as described in paper I. The subject traced out her own curve by continually reversing a motor driving a photometric wedge so that the test light alternated between being seen and not seen; thus the tracing appeared as a saw-tooth curve oscillating about the curve of true threshold. In the normal subject the cone branch of the dark-adaptation curve can be distinguished from the rod branch by changing the wave-length of the test flash. This was shown in Fig. 1 of paper I, where the test flash alternated each minute between yellow (580 mμ) and green (520 mμ) of equal scotopic intensity. The rods, therefore, could not distinguish between the two test lights and the rod curve was smooth, but the cones were some three times more sensitive to the yellow than the green. Consequently, the cone threshold jumped half a log. unit at each change of light, and the cone curve presented a castellated appearance.

Now we have seen in paper II that our photanope, D.M., has a cone of spectral sensitivity rather similar to that of normal cones and very different from that of normal rods; thus her dark-adaptation curve traced by the alternating test-colour technique would be expected to show a smooth rod branch and a castellated cone branch. Figure 2 shows that this is the case. The curves are the actual tracings made by the photanope in two experiments from the same sitting, following different bleaching exposures. Each curve exhibits an initial castellated cone branch where the yellow flash is far more effective than the green, and where very little fall in threshold is seen. The second branch *per contra* shows no castellation in any part (though the alternation of flash colour continued throughout the whole curve) and the fall in threshold for a green flash covered about 6 log. units.

If we are correct in interpreting the tracings as cone and rod branches, we should expect the first branch to show a Stiles–Crawford effect, and the second branch to show none. This, in fact, was confirmed in a separate experiment.

The subject was aligned in the apparatus shown in Fig. 1 of paper II, with the background field abolished by an opaque screen at F_1. After full bleaching the fairly constant cone threshold was measured during the first 5 min of dark-adaptation with the point of entry alternately through the centre of the pupil or through the edge, as at A or B (inset) Fig. 2 of paper II. It was found that there was a Stiles–Crawford effect during the first few minutes, though only about 0·2 log. units in magnitude (consistent

with 0·5 for the double value found in paper II). Upon the rod branch, on the other hand, no Stiles–Crawford effect was detectable.

The great range of dark-adaptation shown by the rods in Fig. 2 suggests a possible source of error. If the retinal area exposed to the bleaching light is limited (it was a 20° circle in our case), the rods outside this area will not be bleached, and hence will remain fairly dark-adapted and capable of being excited by stray light if sufficiently intense. Now it will be observed that when rods first appear in Fig. 2 their threshold is raised

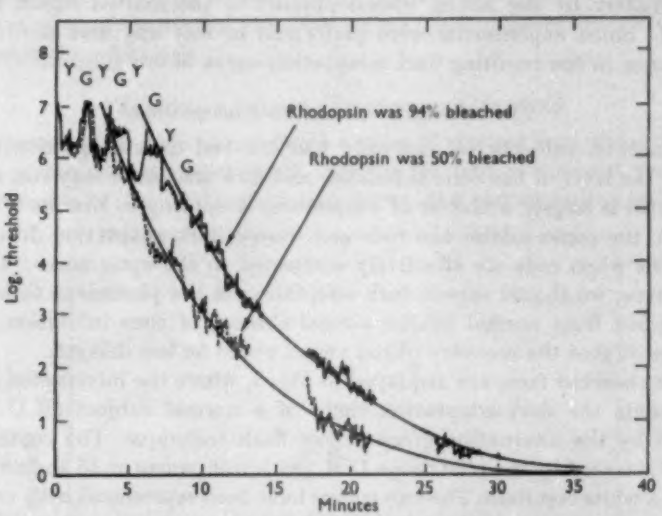

Fig. 2. Dark-adaptation in photanope following 94 and 50 % bleachings. Alternate yellow, Y, and green, G, test flashes throughout the whole curve.

about a million times above the final value, so 1/100,000 part of this test intensity scattered into the periphery would probably suffice to excite the rods there. And indeed attention was drawn to this by our subject who said, 'During the first few minutes, I can see out of the corner of my eye that the light is flashing, but I suppose that you only want me to say I see it when I see it where it really is.'

In a pure cone-free retina an error of this kind would produce an early branch resembling that of Fig. 2, but without castellation, and it might be interpreted as due to receptors which contain rhodopsin but regenerate very fast—'rhodopsin cones' or 'daylight rods'. This effect of scatter cannot account for the early branch of Fig. 2, for the receptors here showed the Stiles–Crawford effect and had a spectral sensitivity very different from that of rhodopsin, and hence could not have been peripheral rods;

moreover, the possibility of error was detected and set aside by the quick intelligence of our subject. I do not wish to suggest that this error has in fact misled any former workers, but when dark-adaptation in the photanope appears to reveal 'daylight rods', the possibility of scatter should clearly be borne in mind and carefully excluded.

One way to do this is to reduce the sensitivity of all the rods in the retina by giving a preliminary bleach from a large milk-glass screen at moderate illumination placed very close to the eye. This is then followed immediately by the strong bleach applied to the limited region to be tested. Some experiments were performed in this way and showed no difference in the resulting dark-adaptation curve of our subject.

The relation to normal dark-adaptation

In normal subjects we can only plot the rod dark-adaptation curve below the level of the cone threshold, and how the curve may run above this level is largely a matter of conjecture. According to Elenius & Heck (1957), the cones inhibit the rods and during dark-adaptation delay the moment when rods are effectively connected to the optic nerve. If this were true, we should expect dark-adaptation in the photanope to be distinguished from normal by the virtual absence of cone inhibition, with the result that the recovery of rod vision would be less delayed.

The observed facts are displayed in Fig. 1, where the interrupted curve represents the dark-adaptation curve of a normal subject (M.G.F.F.) tested by the alternating green-yellow flash technique. The continuous curve is traced by the photanope D.M. (with rest pauses at 15 and 20 min), using a white test flash. The two curves have been reproduced with vertical displacements so that they coincide at full dark-adaptation. Both subjects had a similar bleaching exposure (97 % bleach) and both showed similar rhodopsin regeneration, as indicated in Fig. 1 by the black and white circles. The dark-adaptation curve of the normal subject shows the castellated cone branch for the first 20 min, at which point the photanope curve is met. Thereafter the two curves march together. This does not at all support the idea of rods being inhibited by cones, but it is precisely what should happen if rods and cones are independent mechanisms and if the more sensitive in any situation alone determines the threshold, as Stiles (1949) has established for increment thresholds.

The evidence upon which Elenius & Heck (1957) based their conclusions was derived, not from visual thresholds but from e.r.g. measurements upon photanope and normal subjects. This work we have attempted to repeat and shall discuss elsewhere; here we simply state that we have not confirmed their observations.

It seems reasonable to conclude that in the photanope both rods and

rod vision are normal. For not only is the regeneration of rhodopsin the same in photanope and normal subjects (black and white circles, Fig. 1), but photanope and normal rod thresholds also coincide throughout the whole range where normal rods can be measured. It seems likely, therefore, that the thresholds of normal rods, where they cannot be directly observed (namely, above the level of the cones) are also given by the full sweep of the photanope curves as shown in Figs. 1 and 2. The relation, then, between log. threshold and the fraction of rhodopsin present which, for the photanope, is plotted over a large range in Fig. 1, may be considered as the relation of the normal rods; and we may now measure the curves and find what the relation is.

The relation between rhodopsin and threshold

Suppose that we draw across Fig. 1 a series of vertical lines, the ordinate values of the rhodopsin curve will give the fraction of pigment present, the ordinates of the dark-adaptation curve, the corresponding value of

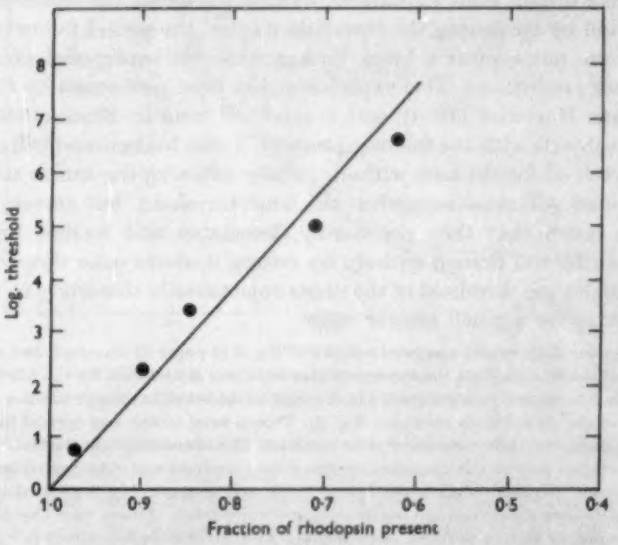

Fig. 3. Relation between log. threshold and fraction of rhodopsin present in photanope.

log. I/I_0—the logarithm of the threshold when this is expressed as a multiple of the fully dark threshold. In Fig. 3 these two values have been plotted one against the other, and a reasonably linear relation is found. This is the relation which in paper I it was argued should occur, and which has been

found by Dowling & Wald (1960) to exist between the rhodopsin content and log. e.r.g. threshold in the rat.

The lateral displacement between the two curves of Fig. 2 is consistent with this relation between rhodopsin and threshold. The bleaching exposures in each case lasted 30 sec and amounted to 6·8 and 7·4 log. td sec, respectively. Now in a previous investigation (Rushton, 1956) it was shown that 6·8 log. td sec leaves $\frac{1}{2}$ the rhodopsin unbleached. Therefore, 7·4 log. td sec, which is 4 times this energy, will leave $(\frac{1}{2})^4$ or about 6% unbleached. But from Fig. 1 it is seen that rhodopsin takes 4 min to regenerate from the 6% to the 50% level. Hence the two curves of Fig. 2 should be laterally separated by an interval of 4 min. In fact, the curves are not quite the same shape, being separated by about 3 min in the upper part and 5 min in the lower part, but the displacement is of the right order of magnitude.

DARK-ADAPTATION AND INCREMENT THRESHOLD

We may obtain some important information about the nature of dark-adaptation by measuring the threshold flash in the period following light-adaptation, not against a black background, but superposed upon fields of various luminances. This experiment has been performed by Crawford (1947) and Hattwick (1954), and in modified form by Sloan (1950), upon normal subjects with the following results. A dim background will raise the final threshold for the rods without greatly affecting the cones; a brighter background will raise somewhat the cone threshold, but elevate that of rods so much that they are hardly detectable and further brightness abolishes the rod branch entirely by raising it above cone threshold.

The high cone threshold of the photanope permits this study to be made upon rods over a much greater range.

The increment threshold equipment shown in Fig. 1 of paper II was used, and a region 7° parafoveal examined. First the increment threshold was determined for the whole range of background fields, with a white flash which could be made bright enough to show some very high threshold cones (black triangles, Fig. 5). Then a total bleach was applied in the usual way and dark-adaptation measurements initiated. The increment light flashed continually and the subject moved the interposed wedge until threshold was obtained. When satisfied she remained in position upon the biting block and snapped her fingers, the time was immediately noted and then the wedge setting was recorded. Filters were changed in flash and background beams between each reading so that five dark-adaptation curves were simultaneously investigated, each with a different background field. Finally, after 45 min, the whole increment-threshold curve was redetermined (dots) and found to agree with the initial values.

Dark adaptation against a fixed background light

The five dark-adaptation curves are plotted in the usual way as a family of curves in Fig. 4. With the exception of the upper curve at a background of 2·4 log. td, which lies close to rod saturation (paper II; Aguilar & Stiles,

1954), the curves show the features already clear from the results of previous workers. The dark-adaptation curves all coincide for the first 14 min. and then, one by one, suddenly stop their increase in sensitivity and rather abruptly level out at a value dependent upon the background intensity.

There has been a tendency to explain this in terms of the bleaching of rhodopsin by the background light, which thereby opposes regeneration and brings the pigment into equilibrium at a level somewhat short of full regeneration and, hence, of full sensitivity. There are strong arguments against this explanation.

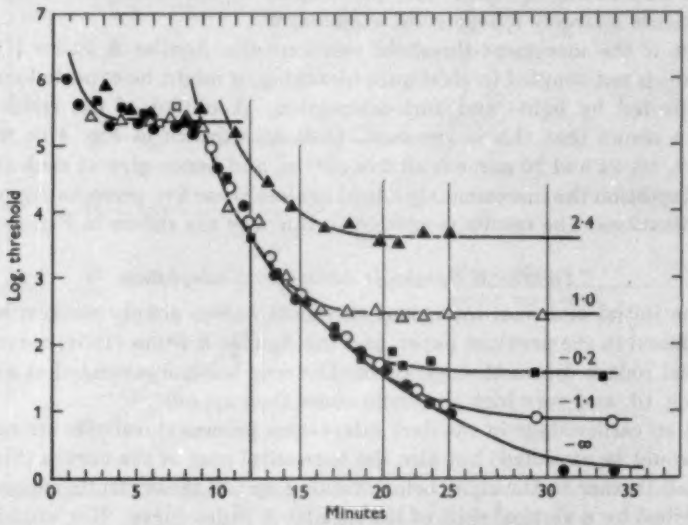

Fig. 4. Dark-adaptation curves of photanope against background fields of luminance shown by number (log. td), against each curve.

From Fig. 4 it appears that a background field of −1 log. td raises the increment threshold by 1 log. unit. If this is due to bleaching, we can see from Fig. 1 that about 4 % of rhodopsin must have been bleached, since this raises the dark-adapted threshold 1 unit above the absolute value. But, −1 log. td corresponds to a retinal illumination of about 1 quantum (500 mμ) incident per rod per second, and even in complete absence of regeneration such a light would take 4 months to bleach away the required 4 %, accepting the photosensitivity measurements of Dartnall, Goodeve & Lythgoe (1938) for rhodopsin in solution, which have been shown to apply approximately to the living human eye (Rushton, 1956).

But a sudden change of background field causes the new increment

threshold to be established, not in the matter of months nor even of minutes, but within a second or so. It cannot, therefore, depend upon the bleaching and regeneration of rhodopsin.

The dark-adaptation threshold obtained against various backgrounds depends, in fact, upon the background intensity, but not upon the time (greater than a second or two) through which this background has been maintained. In sharp contrast to the 20–30 min delay required to reach full dark-adaptation with any background is the mere 2 sec wait needed when changing back and forth between various fields at every stage of dark-adaptation, as experienced in the determinations of Fig. 4. Increment thresholds, therefore, are not coupled to the rhodopsin level, but constitute a largely independent mechanism.

But if the increment-threshold relation—the Aguilar & Stiles (1954) curve—is not coupled to rhodopsin bleaching, it might be expected not to be affected by light- and dark-adaptation. A re-plot of the results of Fig. 4 shows that this is the case. Ordinates drawn in Fig. 4 at times 12, 14, 20, 24 and 30 min cut all five curves, and hence give at each stage of adaptation the increment threshold against these five given background illuminations. The results re-plotted in this way are shown in Fig. 5.

Increment thresholds during dark adaptation

The initial and final increment threshold curves simply confirm what was found in the previous paper, and the Aguilar & Stiles (1954) curve for normal rods is drawn through them. The rods become saturated at about 2·7 log. td, and very high threshold cones then appear.

At an earlier stage in the dark adaptation process thresholds are raised (as would be expected) but also the horizontal part of the curves (Fig. 5) stretch further to the right before turning up; so these results cannot be described by a vertical shift of the Aguilar & Stiles curve. Nor would the curve be expected to shift vertically. For if bleaching renders the rods less able to generate a signal, this incapacity should affect equally both flash and field. So if the curve must move upwards 2 units because the flash is so ineffective, it must also move to the right 2 units because the field is equally ineffective. The Aguilar & Stiles curve, therefore, should be displaced upwards and to the right along the 45° line. The family of curves drawn in Fig. 5 is, in fact, the Aguilar & Stiles curve so displaced and it is seen to fit the results for all backgrounds below near-saturation at all stages of adaptation.

But the saturation barrier was never crossed by the rods. Specific investigation of this point confirmed the fixity of that vertical saturation line. For instance, the uppermost square (Fig. 5) was compared with the same flash upon a background 2·8 log. td—a point just the other side of

the saturation line. In the dark-adapted state, the square would be seen and the other point would not. Raising the threshold 10,000 times by light-adaptation did not change this result. Though the Aguilar & Stiles curve shifted 4 log. units up and to the right, the saturation line did not shift at all. To the left of the line the flash was seen; to the right, it was not.

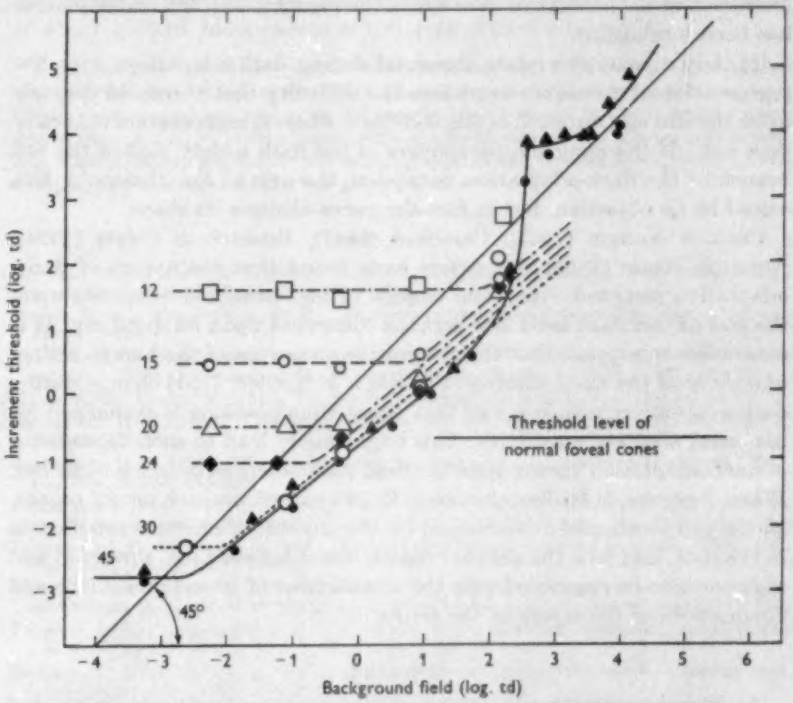

Fig. 5. Increment threshold curves at various stages of dark-adaptation derived from re-plotting the results of Fig. 4. ▲, initial increment threshold relation. Number against each curve shows number of minutes of dark-adaptation.

DISCUSSION

The experiment of Fig. 1 confirms by direct measurement conclusions derived by argument in paper I, and establishes a simple linearity between the amount of rhodopsin still unregenerated and log. threshold for the perception of a test flash. Yet it is important to bear in mind that this simplicity is the result of restricting the range of our investigations, and that in two ways.

(*a*) Though after *strong* bleaches the rod branches of adaptation curves are always the same shape, so that they may be made to coincide by

lateral displacement, this is not the case after weak bleaches. As Winsor & Clark (1936), Hecht, Haig & Chase (1937) and many others have pointed out, rod curves which appear early return quickly. That complication is here avoided by investigating the consequences only of substantial bleaches where rods do not appear early. But this constitutes an important limitation of the conditions over which the pigment–log. threshold relation has been established.

(*b*) Any attempt to relate threshold during dark-adaptation with the regeneration of rhodopsin must face the difficulty that threshold depends upon the size and duration of the test flash, whereas regeneration naturally does not. If changing the parameters of the flash simply shifted the rod branch of the dark-adaptation parallel to the axis of log. threshold, this would be no objection, but in fact the curve changes its shape.

Craik & Vernon (1941), Crawford (1947), Rushton & Cohen (1954), Arden & Weale (1954) and others have found that the extent of dark-adaptation recorded—the total change of log. threshold—depends upon the size of test flash used and perhaps somewhat upon its duration. It is reasonable to suppose that the integrative properties of the human retina, like those of the cat's (Barlow, FitzHugh & Kuffler, 1957) change during dark-adaptation in such a way that space–time precision is exchanged for increased absolute sensitivity. This might easily lead to such dependence of dark-adaptation curves upon the flash parameters as in fact is observed. What, however, is far less obvious is the way in which such neural organization can be uniquely determined by the amount of unregenerated opsin in the rods, and how the simple relation found between log. threshold and pigment is to be reconciled with the complexities of lateral inhibitions and summations in the layers of the retina.

SUMMARY

1. Visual pigments were measured in the eye of one incomplete rod monochromat by retinal densitometry. No visual pigment was detected upon the fovea, but in the periphery rhodopsin was found to be normal both as to amount and the rate of regeneration.

2. The absence of nearly all cones in the periphery permitted the rod branch of the dark-adaptation curve to be followed over 6 log. units of threshold intensities. The curve coincided with that of the normal subject when this appeared below the cone branch (Fig. 1), thus the monochromat's curve probably represents the normal rod threshold where this cannot be measured above the cones.

3. At each stage of dark-adaptation there is a linear relation between log. threshold and the amount of rhodopsin still unregenerated (in the particular conditions investigated).

4. Measurements of increment thresholds upon various background fields at different stages of dark-adaptation show a simple relation. Over most of the range the effect of light-adaptation could be imitated by placing a suitable neutral filter in front of the eye, thus raising the threshold equally for flash and field.

5. But the luminance of background which saturates remains constant at about 1000 td, independent of the state of dark-adaptation.

It is a pleasure to express my great indebtedness to Drs M. G. F. Fuortes and R. D. Gunkel who have modestly refused to allow their names to appear as joint authors of this paper though they co-operated fully in all the experiments. I should like to thank the many authorities at N.I.H. for inviting me to come, and for placing every facility at my disposal. And I am especially grateful for the unfailing support and encouragement of Dr L. von Sallmann in whose department I was privileged to work.

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PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

LONDON MEETING

14 *January* 1961

Expired air resuscitation

By L. M. Croton and W. R. Davies. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

The method of expired air resuscitation (oral resuscitation) at present advocated consists of the following stages:

(1) Lay the asphyxiated victim in the supine position, if possible on a table or on ground higher than that on which the rescuer is standing.

(2) Clear his mouth of any obstruction.

(3) Place a folded coat or other support under his shoulders so that the head falls well backwards.

(4) Kneel by the side of the victim's head, or stand by the side of the table opposite his head.

(5) Press his head to the fully extended position with one hand, with the other draw his jaw well forward, and maintain this extension throughout. The combination of these two movements should ensure that the airway is fully patent.

(6) After taking a full inspiration, the rescuer places his mouth over the victim's mouth and breathes strongly into his lungs, ensuring that the victim's nasal orifice is blocked off by pinching the nose. The rescuer then turns his head away from the victim whilst passive expiration takes place, then he takes another breath and repeats the cycle.

(7) This cycle is repeated every 3–5 sec, breathing strongly for an adult and lightly blowing for a child.

The problem of instruction of First Aid personnel and rescue workers is of major importance. Several training manikins have been produced and instructional films are at present being used to illustrate the physiological principles governing the application of this method of artificial respiration.

Blood-pressure measurement without observer variability

By W. W. Holland and H. S. Wolff. *Department of Epidemiology, London School of Hygiene and Tropical Medicine, and Bioengineering Laboratory, Division of Human Physiology, National Institute for Medical Research, London, N.W. 3*

The recording accuracy of a normal sphygmomanometer for the measurement of the blood pressure of large numbers of subjects is limited by the variability of the rate of cuff inflation and deflation, and the necessity for the observer to concentrate not only on hearing the appearance and change of the Korotkow sounds but also on recording the level of a moving column of mercury. Recording to the nearest 5 mm mark is of little use in large surveys as the resultant frequency distribution curve is irregular. Both this procedure and reading to the nearest 2 mm leads to marked digit preference (Rose, personal communication).

The apparatus illustrated aims at eliminating these sources of observer error. Pressure is recorded on three standard mercury manometers which, during the process of taking the pressure, cannot be seen by the observer. The latter is able to stop the descent of each column at the appropriate moment by closing a magnetic valve. The observer thus merely listens to the arterial sounds, presses a button when the sounds change, and can then subsequently read the pressures on static mercury columns. The rate of cuff inflation and deflation is controlled by appropriate valves so as to be constant throughout.

Lifting and carrying stretchers. (Film)

By G. P. Crowden. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C. 1.*

The film shows that the heavy muscular work of stretcher bearers may be facilitated by:

(1) Positioning the casualty so as to equalize the load on the bearers.

(2) Placing one foot in front of the other, and bending the knees when lifting or lowering the loaded stretcher.

(3) Using shoulder-yoke slings which eliminate static effort and strain on the hands and arms. This type of sling greatly reduces energy expenditure in carrying (Rao, 1953), retards the onset of fatigue and so makes longer carries possible.

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Measurement of pulmonary resistance by the airflow interruption method

By J. I. PALMER. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

How does a man twist in the air? A study of rotations generated when falling or diving into water. (Film)

By D. A. MCDONALD. *Department of Pharmacology, Medical College of St Bartholomew's Hospital, London, E.C.* 1

The effect of immersing the human body in water on pulmonary diffusion and capillary blood volume.

By A. R. GUYATT, FAITH NEWMAN, J. I. PALMER and M. L. THOMSON. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

Alveolar carbon dioxide expiratory plateau by fast katharometer

By A. R. GUYATT. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

Tissue culture of the rat anterior pituitary; some observations on outgrowing cells

By J. K. BURNS.* *H.E.R.T. Tissue Culture Laboratory, Department of Biochemistry, Institute of Physiology, University of Glasgow*

Investigations concerning the cytology of cultured anterior pituitary cells have given variable results, acidophils, basophils or 'undifferentiated' cells having been described by previous authors. Vesiculate chromophobes, which develop after thyroidectomy, adrenalectomy or gonadectomy in experimental animals (Severinghaus, 1938) have not been observed in outgrowing cells of pituitary explants, in cultures maintained for 2 weeks (Guillemin & Rosenberg, 1955). The absence of normal hormonal influences could be expected to result in the appearance of such cells *in vitro*.

In the present experiments, anterior pituitary explants from 2-month-old rats were cultured in plasma clots, on coverslips in modified roller tubes. The fluid medium (5 ml.) consisted of 85% Hanks's balanced salt solution (Paul, 1959) or Waymouth's medium (Waymouth, 1956) with 10% calf serum and 5% chick embryo extract. Media were changed twice weekly, and cultures were maintained for 2 weeks. Series of cultures of chick heart fibroblasts, grown as hanging-drop preparations for 2 days,

* Present address: Department of Physiology, University College, Galway, Eire.

served as controls for staining procedures. Cultures were stained with the periodic acid Schiff, alcian blue, toluidine blue, Sudan IV, Sudan black B and coupled tetrazolium techniques (Pearse, 1960) and a Mallory method (Carleton & Leach, 1957).

Vesicles were present in almost all outgrowing cells. The outer rims of these stained with the periodic acid Schiff method. They did not react with alcian blue, nor did they show metachromasia with toluidine blue. The coupled tetrazolium reaction for protein stained the cytoplasm, but not the vesicles. These were not demonstrated by Sudan IV, but Sudan black B gave a positive reaction for complex liquid. The Mallory method showed unstained vacuoles in practically all cells. Treatment of cultures with absolute alcohol for 90 min at 56° C resulted in a negative periodic Schiff reaction and absence of staining with Sudan black B. These results are consistent with the Schiff positive material being formed of glycolipid. The composition of the material within the vesicles has, however, not yet been established.

The morphology of these cells, as shown by the Mallory method, seems similar to that of vesiculate anterior pituitary cells observed by Severinghaus (1938) in adrenalectomy, thyroidectomy and gonadectomy; by Pearse (1949) in senile patients and by Guyer & Claus (1932) following cancer transplantation in rats. Further experiments are being planned, with the object of investigating this similarity.

This work was carried out during the tenure, by the author, of a Council of Europe Fellowship.

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The effect of cyclical heating of the front of the trunk on the rate of sweat production from the forearm

By D. F. Brebner and D. McK. Kerslake. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

The experiments to be described were intended to test for the existence of thermoreceptors in the skin of the trunk contributing to the control of the rate of sweat production elsewhere.

The skin of the front of the trunk, from the clavicles to the pubis, was exposed to radiant heat, the intensity of which was varied sinusoidally with periods of 8–70 sec. The mean difference between the maximum and minimum radiation intensity, measured in the coronal plane, was 310 kcal/m^2 hr. The skin reflected about 20 % of the incident radiation. A capsule ventilated with dry air was applied to the volar surface of the right forearm (not exposed to the radiation) and the sweat production was measured continuously by means of an infra-red gas analyser through which the effluent air was passed.

Continuous records of the output of the analyser showed large, apparently random, fluctuations on which were superimposed peaks corresponding in frequency with the stimulus. In order to examine the time relations of the response more accurately, the mean sweat rate during each tenth of a large number of cycles was recorded (usually 10 min continuous running). The curves so obtained showed clear maxima and minima, the phase lags of which were measured with respect to the radiation cycle. The relation between phase lag and frequency was linear, suggesting that the time lag between maximum radiation and maximum sweat rate and between the respective minima was constant at 9·0 sec. Calibration of the system by injection of sinusoidal changes of humidity into the capsule effluent gave similar results, the time lag being 7·4 sec. The sweat response was not abolished by occluding the circulation to the arm.

The results suggest that the forearm sweat rate follows the stimulus with a delay of about 1·6 sec, so that the pathways involved must be neural. The response is related to peak radiation intensity, which occurs before the skin temperature reaches its peak. The stimulus is therefore probably either rate of change of skin temperature or rate of heat exchange. The response does not appear to be secondary to reflex circulatory changes in the forearm skin.

Carotid chemoreceptor response to high carbon monoxide tension

By N. Joels and E. Neil. *Department of Physiology, Middlesex Hospital Medical School, London, W.* 1

The combination of as much as 80 % of the blood haemoglobin with CO causes no carotid chemoreceptor discharge in cats breathing 1–2 % CO in air, providing that the arterial pO_2 be not lowered (Duke, Green & Neil, 1952). The glomus cells normally betray a high oxygen usage (9 ml./100 g/min). Their blood flow of 2000 ml./100 g/min (Daly, Lambertsen & Schweitzer, 1954) is, however, so high that they have little difficulty in extracting their large oxygen requirements without causing much fall in the local blood pO_2. Anoxic anoxia disrupts their oxidative metabolism,

whereupon the chemoreceptor nerves display a vigorous impulse traffic. Cyanide also causes chemoreceptor discharge, and Anitschkov (1951) has assumed that the drug acts by inhibiting cytochrome oxidase. Cyanide, however, inhibits many of the enzymes which aid carbohydrate metabolism. Only carbon monoxide exerts a *specific* inhibition of cytochrome oxidase (Dixon & Webb, 1958). The affinity of cytochrome oxidase for CO is less than that for O_2. Thus it is necessary to use high CO tensions locally in the glomus circulation in order to ascertain whether chemoreceptor discharge ascribable to cytochrome oxidase inhibition can be aroused.

Two sets of experiments were performed on cats anaesthetized with pentobarbitone.

(1) 1–2 ml. of blood (pCO = 300–500 mm, pO_2 = 150 mm, pCO_2 = 40 mm Hg) was injected locally into the carotid glomus circulation. Transient hyperpnoea and hypertension which occurred was abolished by cutting the relevant sinus nerve.

(2) Electroneurography of chemoreceptor fibres showed that vigorous impulse activity was aroused by the perfusion of solutions equilibrated with pCO 300–500 mm Hg and normal pO_2 and pCO_2.

The inhibition of cytochrome oxidase thus does mimic the effect of anoxic anoxia on the glomus cells. It must be stressed that CO is used here as a biochemical tool; ordinarily in CO poisoning the tissue pCO is far too low to exert such histotoxic effects.

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The effect of linear acceleration on the response to angular acceleration in man

By A. J. BENSON and T. C. D. WHITESIDE. *Royal Air Force Institute of Aviation Medicine, Farnborough, Hants*

Both Ledoux (1949) and Gernandt (1950) have shown in animals that the response pattern of ampullary receptors is modified by linear acceleration. To see if this observation was applicable to man, experiments were carried out in the human centrifuge by which a reproducible rotational stimulus could be combined with different linear accelerations.

Eight subjects each experienced two runs near the centre, and two at the end of the centrifuge arm whilst in a fixed seat, with head vertical and facing the centre of rotation. The rotation was steady at 25°/sec for 2 min;

accelerating 10°/sec² for 8 sec; steady at 105°/sec (3·1 *g*) for 1 min; decelerating 10°/sec² for 8 sec; steady at 25°/sec for 2 min. The duration of the sensation of turning was measured, and by making use of the corneo-retinal potential, recordings of horizontal nystagmus were made whilst the eyes were closed.

There was no consistent significant change in sensation times, which varied greatly, possibly because of the superimposed tilting sensation experienced during rotation at the end of the arm. The angular velocity of the slow phase of nystagmus rose during angular acceleration, then decayed exponentially. For this decay the regression equation was calculated for each angular stimulus. Analysis of variance of the slopes and intercepts revealed that the rate of decay of nystagmus was significantly increased ($P = 0{\cdot}001$) only in the presence of high linear acceleration(3·1 g).

Possible explanations of this reduced response are that the cupula is affected by linear as well as by angular accelerations, or that the nystagmus was modified by impulses from utricular or saccular maculae.

We are grateful to Miss Helen Ferres for statistical analysis of the data.

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A comparison between athletes and non-athletes in oxygen consumption and pulmonary diffusion at near-maximal exercise

By FAITH NEWMAN, B. F. SMALLEY and M. L. THOMSON. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

Bannister, Cotes, Jones & Meade (1960) reported that values of pulmonary diffusing capacity at given levels of exercise were systematically higher for athletes than for non-athletic subjects ($P < 0{\cdot}05$).

In the present investigation eleven medium and long-distance runners, with one exception all of international class, were compared with nine healthy non-athletes closely matched with the athletes in body size and age. Measurements of pulmonary diffusion (k_{CO}) by breath-holding method (Ogilvie, Forster, Blakemore & Morton, 1957), oxygen consumption ($\dot{V}_{O_2}$) and pulse rate were made on a bicycle ergometer at rest and at three levels of exercise, in one of which the subjects attempted to reach their maximum. The index k_{CO} is preferred to D_{CO}; it is the time constant of the exponential decay of CO concentration during breath-holding and is proportional to D_{CO} if, as in the orthodox method, measurements are made at maximum lung volume (McGrath & Thomson, 1959).

The most obvious difference between the two groups was the significantly higher maximal $\dot{V}_{O_2}$ achieved by the athletes (mean for athletes, 3·71 l./min, and for non-athletes 2·67 l./min; $P < 0·01$). The mean maximum values of k_{CO} corresponding to these values of $\dot{V}_{O_2}$ were 7·53 min^{-1} and 6·56 min^{-1} for athletes and non-athletes respectively. The corresponding mean pulse rate of 176/min for athletes was significantly lower than that of 225/min for non-athletes ($P < 0·001$).

It was found, in agreement with Bannister *et al.* (1960), that the regression for k_{CO} on $\dot{V}_{O_2}$ ($k_{CO} = 4·78 + 0·818\ V_{O_2}$ for athletes) was higher in athletes than in normals, although the slopes of the two lines were virtually parallel. The difference between the two lines of approximately 0·35 min^{-1} k_{CO} was not however significant ($0·1 > P > 0·05$).

As expected in these two matched groups the mean total lung capacity and its subdivisions did not differ significantly between athletes and non-athletes. The mean forced expiratory volume for 1 sec (F.E.V. (1)) was 4·76 l. for athletes and 5·01 l. for non-athletes; this difference was not significant and the ratios F.E.V./V.C. (86·7% and 87·3% respectively) showed even closer agreement.

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Simultaneous acclimatization to heat and cold in man

By E. M. GLASER* and R. J. SHEPHARD. *Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts*

There is evidence that people can repeatedly adapt to successive 3-day periods at about 4° and 0° C (Glaser, 1949) and that a hand can be adapted at one and the same time to immersions at 47° and 4° C (Glaser, Hall & Whittow, 1959). The present experiments attempted to induce simultaneous acclimatization of the whole body to heat and cold.

Fifteen men spent 3 hr every morning at 35° C (dry bulb) and 28° C (wet bulb), and 3 hr every afternoon at about 3° C for 11 days. They wore shorts in the hot room and indoor clothes in the cold. An index of comfort suggested that both environments became progressively more comfortable. In the cold room the skin of the hands and fingers became progressively

* Address: The London Hospital Medical College, Turner Street, E. 1.

warmer, rising from a mean of 11·9° C in the first test to 15·5° C in the eleventh, and the blood flow through the hand tended to increase; mouth temperatures were inaccurate because of shivering and they showed no significant improvement. In the hot room the mean mouth temperatures became gradually less, falling from 37·2° C in the first test to 36·8° C in the last, but the skin temperatures showed no clear tendencies, perhaps because the experiments were carried out after an exceptionally hot spell in 1959.

Responses of the blood pressure and of the heart rate to immersion of a hand at 47° C and at 4° C (Glaser *et al.* 1959) were tested before and after the eleven exposures to heat and cold. At the end of the experiment there was a significant diminution of the responses both to warming and to cooling, which suggested that warming and cooling of the whole body could inhibit responses to localized warming and cooling.

There was a fall of the mouth temperature on the day after the eleventh exposure to heat and cold at the time when the subjects would have been entering the hot room, and there was a rise of the mouth temperature at the time when the subjects would have been entering the cold room. This could be further evidence that diurnal rhythms of temperature are influenced by past activities (Glaser, 1957).

The fact that the present experimental subjects became acclimatized to both heat and cold at the same time, tends to support previous evidence that acclimatization may depend, at least in part, on the way in which the central nervous system responds to thermal stimuli.

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A comparison between body size and lung function of swimmers and normal school children

By Faith Newman, B. F. Smalley and M. L. Thomson. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

Efficiency of lung ventilation of thirty leading British swimmers (fifteen of each sex) of mean age 16·1 (13–17) was compared with that in a control group of nineteen school children (ten males and nine females), average age 16·7 (14–18) from a London County Council School.

Table 1 shows means, standard deviations and probability levels for the significance of the difference between means of the physical characteristics, weight, height and stem height, and measurements of vital capacity (V.C.),

forced expiratory volume (F.E.V. (1)) and maximum expiratory pressure (M.E.P.).

The M.E.P. is measured by calibrated aneroid-type pressure gauge and is the maximum static force of which the chest bellows is capable. The F.E.V. (1) is the maximum volume which can be expelled in 1 sec from full inspiratory position and is measured by special spirometer. Since resistance is defined as pressure/flow, the ratio M.E.P./F.E.V. (1) should give an index of total pulmonary resistance. The index M.E.P. × F.E.V. has not, so far as is known, been previously used: it is a measure of power (pressure^2/resistance).

TABLE 1. Physical characteristics and lung function measurements

		Weight (kg)	Height (cm)	Stem ht. (cm)	V.C. (l.)	F.E.V. (1) (l.)	M.E.P. (mm Hg)	M.E.P./ F.E.V.	M.E.P. × F.E.V.
Swimmers (M)	Mean	69·1	176·4	93·5	5·00*	4·85	100·0*	22·90*	534·0*
	S.D.	7·51	6·84	4·9	0·39	0·73	18·52	4·39	70·53
Non-swimmers (M)	Mean	61·6	168·9	83·8	4·80	4·09	75·1	18·56	308·9
	S.D.	11·60	8·40	2·5	0·65	0·54	11·90	3·32	72·40
	Difference between means	7·5	7·5	9·7	1·10	0·76	33·9	4·34	225·1
	Probability	< 0·1	< 0·05	< 0·001	< 0·001	< 0·02	< 0·001	< 0·02	< 0·001
Swimmers (F)	Mean	59·3	164·1	88·2	4·55	3·79	76·0	20·50	285·0
	S.D.	6·17	6·03	4·5	0·49	0·42	22·89	7·50	83·50
Non-swimmers (F)	Mean	51·4	160·6	78·5	3·34	2·82	50·7	18·10	146·2
	S.D.	7·00	6·30	4·7	0·49	0·50	11·20	3·36	49·50
	Difference between means	7·9	3·5	9·7	1·21	0·97	25·3	2·40	138·8
	Probability	< 0·02	< 0·2	< 0·001	< 0·001	< 0·001	< 0·01	< 0·4	< 0·001

* One or two measurements were beyond the capacity of the instruments.
All volumes given at B.T.P.S. V.C. = vital capacity; F.E.V. (1) = 1 sec forced expiratory volume; M.E.P. = maximum expiratory pressure.

Mean weight, height and stem height were greater in swimmers, the difference in stem height being highly significant. The mean ponderal index reciprocal (height/weight$^{\frac{1}{3}}$) was identical in the two groups. The V.C., F.E.V. (1), M.E.P., F.E.V. (1) × M.E.P., and M.E.P./F.E.V. (1) were higher in swimmers of both sexes; the differences were significant with the exception of M.E.P./F.E.V. (1) in females.

This work was supported by a grant from the Medical Research Council. Thanks are due to Mr J. Atha, lecturer at Loughborough College of Physical Education, who measured the weight, height and stem height of the swimmers.

Non-uniformity of alveolar capillary diffusion in the human lung

By A. R. GUYATT. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

Ogilvie, Forster, Blakemore & Morton (1957) found that the apparent diffusing capacity of the human lung for carbon monoxide (D_{CO}) decreased

slightly (about 1·6% sec) with increase in breath-holding time (T) from 5 to 14 sec. Jones & Meade (1960) attributed part of this decrease to error in the estimation of mean T and described an improved method. Forster, Fowler & Bates (1954) showed theoretically that such a change in diffusing capacity with T indicated non-uniformity of diffusion throughout the lung.

It has been claimed (Mittman & Burrows, 1959) that diffusion is more uniform at mid capacity than at full inspiration. This is unexpected, because it is known that ventilation is less uniform at mid capacity. Moreover, it has been shown (McGrath & Thomson, 1959) that in normal subjects k_{CO}, the time constant of the uptake of CO, increases when lung volume is reduced at constant T, suggesting an increase in the ratio, area of diffusing surface to volume of the alveoli. The index $k_{CO} = D_{CO} \times$ total pressure of dry gas/volume of lung; it is proportional to D_{CO} if lung volume remains constant.

In the present investigation uniformity of pulmonary diffusion for CO was examined at varying lung volumes and T (7–40 sec), using Ogilvie *et al.* (1957) modification of Krogh's breath-holding method, and the method of timing of Jones & Meade (1960).

In six normal subjects (5 males and 1 female) mean gradient of the (k_{CO}, T) graph for total lung capacity (T.L.C.), and 2/3, 1/2 and 1/3 vital capacity (V.C.), was −0·71, −3·74, −2·82 and −6·60/min respectively. Non-uniformity of diffusion therefore increased as lung volume was reduced.

In two chronic bronchitic patients with emphysema the mean level of k_{CO} and the gradients of the (k_{CO}, T) graphs at T.L.C. and half V.C. range were more alike than in normal subjects and the gradients no longer differed significantly from zero. Possible explanations for these findings are discussed.

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A simple method of preparing gastrin

By E. L. BLAIR, A. A. HARPER, H. J. LAKE, J. D. REED and T. SCRATCHERD.
Department of Physiology, Medical School, King's College, Newcastle upon Tyne 1

Since the work of Edkins (1906) various methods have been described for preparing from the gastric antral mucosa extracts which stimulate acid

secretion by the stomach (Komarov, 1942; Uvnäs, 1945; Harper, 1946; Jorpes, Jalling & Mutt, 1952; Gregory & Tracy, 1959). Some of these methods are elaborate and expensive and many have the disadvantage that the extracts provide variable and low yields of activity per gram of mucosa or are insoluble.

We have for some years been using a relatively crude although simply prepared, soluble, antral extract with a reliable yield, as a starting material for work concerned with the separation of the various gastrointestinal activities which it possesses. The method involves acetone precipitation of the type of aqueous extract described by Edkins. The mucosa is first minced, then homogenized in four volumes of water for $1\frac{1}{2}$ min, and thereafter boiled for 10 min. After cooling it is centrifuged at 2000 *g* for 30 min and filtered first through glass-wool and then through Whatman no. 12 fluted paper during the night, at 5° C. The clear filtrate is precipitated with twenty volumes of acetone and the precipitate is washed with acetone and ether and dried.

The product is a fine white powder which is quite stable and has been kept without evidence of decrease in activity for as long as 4 years. It is fully soluble in water at neutral pH. Active extracts have been obtained from the antra of man, pig, dog and cat (mean yield from the pig = 11·33 mg/g mucosa; S.D. $\pm$ 2·51). When given by intravenous injection to vagotomized, splanchnotomized, anaesthetized cats over a period of 2 min, the yield from 0·5 g of pig antral mucosa, dissolved in 2 ml. of water or 0·9% saline, results in an average output of 0·671 m-equiv HCl (S.D. $\pm$ 0·327; 14 extracts assayed in 48 cats). The response to the same dose can be increased some three times by injecting it slowly in 20 ml. of 0·9% saline over a period of 45 min.

In addition to stimulating acid secretion these extracts result in a sustained, although small, output of pepsin. They also greatly increase the output of pancreatic enzymes with relatively little or no effect on the volume flow of pancreatic secretion. These preparations also have a strong pressor effect upon the tone of the stomach and upper small intestine but are free from any cholecystokinin-like activity.

In the doses that produce these responses, the extracts are without effect on the blood pressure. They contain approximately 0·1 μg of histamine base/mg, and injection of histamine alone in such doses does not produce any of the effects which have been described for the extracts. These effects also persist after atropinization of the assay animal.

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Vagal baroreceptors of the bull-frog

By S. E. DOWNING and R. W. TORRANCE. *Laboratory of Cardiovascular Physiology, National Institutes of Health, Bethesda, U.S.A.*

Neil, Strom & Zotterman (1950) showed that the laryngeal nerve of *Rana temporaria* contains baroreceptor fibres from the pulmonocutaneous trunk but they did not find any baroreceptor fibres from the arterial side of the circulation in the main vagus nerve. Kolatat, Kramer & Mühl (1957) found afferents from the ventricle in this nerve. We have recorded the activity of seven such fibres in experiments on the much larger main vagus nerve of bull-frogs (*Rana catesbiana*, weight about 400 g) anaesthetized with pentobarbitone and with the circulation intact. The right nerve was dissected under paraffin. The spontaneous discharge of single fibres was recorded and the sites of their receptors were determined by direct mechanical stimulation. Blood pressures were altered by occluding vessels or by infusing a dextran solution.

Five fibres came from the base of the ventricle near its junction with the atrium or conus. The discharge of all of them increased when the ventricular pressure was raised. Discharge started before the rise of the aortic pulse and reached an earlier peak. At a high ventricular pressure, three of these fibres had a second peak to the discharge at about the end of ventricular systole.

One fibre came from the conus. The resting discharge came early in ventricular systole. When the pressure in the conus was high, the discharge increased and the peak rate came after the peak of the aortic pressure. One fibre came from the ventral surface of the aorta at its bifurcation and responded to the aortic pressure. Its pathway to the vagus nerve lay beside the conus.

In the main vagus nerve we have also found fibres from the atrium responding during atrial systole; from the mucosa of the oesophagus, the stomach and the trachial bifurcation, responding to touch; and slowly-adapting stretch fibres from the lung.

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Sensitivity of crayfish stretch receptors to temperature changes

By N. VAN GELDER* and K. KRNJEVIĆ. *Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

In general, as stated by Florey (1956) the slow-adapting stretch receptor of the crayfish (*Astacus fluviatilis*) responds to warming by slowing its discharge and to cooling by accelerating. This effect is shown in Fig. 1.

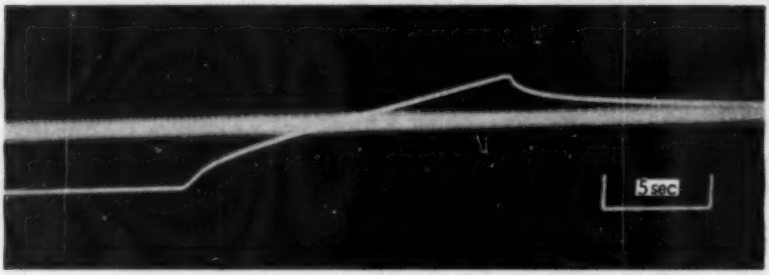

Fig. 1. Impulses recorded from stretch receptor *in situ*. Continuous thermistor trace shows a 2·3° C temperature rise produced with a lamp, from initial level of 13·6° C.

This might suggest that the frequency of firing of the receptor bears a simple relation to the temperature. In fact the relation is quite complex. When the preparation is warmed, there is an immediate transient slowing (just detectable in Fig. 1) followed by a relative or even an absolute acceleration. Marked slowing only becomes apparent as the temperature rises somewhat further. Moreover, there is a clear time dependence. If the temperature is maintained at the new level the receptor shows pronounced adaptation over several minutes, the final rate of firing being relatively little altered. The receptor is much more sensitive to a change in temperature than to its absolute level. Thus a very slow rise or fall of temperature (over several minutes) is not reflected in any change of activity at all.

Florey (1956) has suggested that the thermoelastic properties of the receptor are responsible for some of these effects. If that were so it should be possible to record changes in tensions during warming and cooling, comparable with the tension changes during stretch and release which give rise to similar variations in the rate of firing (Krnjević & van Gelder, 1960). We recorded the tension across an isolated stretch receptor while warming and cooling, but failed to detect any alterations. It is more likely that changes of temperature exert a direct action on the neuronal membrane as in the mammalian spindle (Lippold, Nicholls & Redfearn, 1960). The

* Fellow of the Life Insurance Medical Research Fund.

relative insensitivity of this stretch receptor to the absolute level of temperature is presumably useful in the poikilothermic crayfish.

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Specific sensory structures in hairy skin

By A. IGGO. *Department of Physiology, University of Edinburgh*

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY, 14 *January* 1961

PAGE

Croton, L. M. and Davies, W. R. Expired air resuscitation 1*P*

Holland, W. W. and Wolff, H. S. Blood pressure measurement without observer variability 2*P*

Crowden, G. P. Lifting and carrying stretchers (film) 2*P*

Palmer, J. I. Measurement of pulmonary resistance by the airflow interruption method 3*P*

McDonald, D. A. How does a man twist in the air? A study of rotations generated when falling or diving into water (film) 3*P*

Guyatt, A. R., Newman, Faith, Palmer, J. I. and Thompson, M. L. The effect of immersing the human body in water on pulmonary diffusion and capillary blood volume 3*P*

Guyatt, A. R. Alveolar carbon dioxide expiratory plateau by fast katharometer 3*P*

Burns, J. K. Tissue culture of the rat anterior pituitary; some observations on outgrowing cells 3*P*

Brebner, D. F. and Kerslake, D. McK. The effect of cyclical heating on the front of the trunk on the rate of sweat production from the forearm 4*P*

Joels, N. and Neil, E. Carotid chemoreceptor response to high carbon monoxide tension 5*P*

Benson, A. J. and Whiteside, T. C. D. The effect of linear acceleration on the response to angular acceleration in man 6*P*

Newman, Faith, Smalley, B. F. and Thomson, M. L. A comparison between athletes and non-athletes in oxygen consumption and pulmonary diffusion at near-maximal exercise 7*P*

Glaser, E. M. and Shephard, R. J. Simultaneous acclimatization to heat and cold in man 8*P*

Newman, Faith, Smalley, B. F. and Thomson, M. L. A comparison between body size and lung function of swimmers and normal school children 9*P*

Guyatt, A. R. Non-uniformity of alveolar capillary diffusion in the human lung 10*P*

Blair, E. L., Harper, A. A., Lake, H. J., Reed, J. D. and Scratcherd, T. A simple method of preparing gastrin 11*P*

Downing, S. E. and Torrance, R. W. Vagal baroreceptors of the bull-frog . . 13*P*

van Gelder, N. and Krnjević, K. Sensitivity of crayfish stretch receptors to temperature changes 14*P*

Iggo, A. Specific sensory structures in hairy skin 15*P*

THE JOURNAL OF PHYSIOLOGY, APRIL 1961

VOL. 156, No. 1

CONTENTS

PAGE

FLOREY, E. A NEW TEST PREPARATION FOR BIO-ASSAY OF FACTOR I AND GAMMA-AMINOBUTYRIC ACID 1

KINSEY, V. E. AND REDDY, D. V. N. TRANSPORT OF GLUCOSE ACROSS BLOOD–AQUEOUS BARRIERS AS AFFECTED BY INSULIN 8

GILLESPIE, J. S. AND MACKENNA, B. R. THE INHIBITORY ACTION OF THE SYMPATHETIC NERVES ON THE SMOOTH MUSCLE OF THE RABBIT GUT, ITS REVERSAL BY RESERPINE AND RESTORATION BY CATECHOL AMINES AND BY DOPA 17

FRANK, G. B. THE INFLUENCE OF BROMIDE IONS ON EXCITATION–CONTRACTION COUPLING IN FROG'S SKELETAL MUSCLE 35

BARER, GWENDA R. A COMPARISON OF THE CIRCULATORY EFFECTS OF ANGIOTENSIN, VASOPRESSIN AND ADRENALINE IN THE ANAESTHETIZED CAT 49

OGSTON, A. G. AND SHERMAN, T. F. EFFECTS OF HYALURONIC ACID UPON DIFFUSION OF SOLUTES AND FLOW OF SOLVENT 67

BRADLEY, K. AND SOMJEN, G. G. ACCOMMODATION IN MOTONEURONES OF THE RAT AND THE CAT 75

ASH, R. W. ACID SECRETION BY THE ABOMASUM AND ITS RELATION TO THE FLOW OF FOOD MATERIAL IN THE SHEEP 93

HARKNESS, MARGARET L. R. AND HARKNESS, R. D. THE MECHANICAL PROPERTIES OF THE UTERINE CERVIX OF THE RAT DURING INVOLUTION AFTER PARTURITION 112

EMMELIN, N. SUPERSENSITIVITY OF SALIVARY GLAND CAUSED BY BOTULINUM TOXIN 121

RIZZA, C. R. EFFECT OF EXERCISE ON THE LEVEL OF ANTIHAEMOPHILIC GLOBULIN IN HUMAN BLOOD 128

PIERCE, A. E. FURTHER STUDIES ON PROTEINURIA IN THE NEW-BORN CALF 136

HUXLEY, A. F. AND PEACHEY, L. D. THE MAXIMUM LENGTH FOR CONTRACTION IN VERTEBRATE STRIATED MUSCLE 150

RUSHTON, W. A. H. DARK-ADAPTATION AND THE REGENERATION OF RHODOPSIN 166

FUORTES, M. G. F., GUNKEL, R. D. AND RUSHTON, W. A. H. INCREMENT THRESHOLDS IN A SUBJECT DEFICIENT IN CONE VISION 179

RUSHTON, W. A. H. RHODOPSIN MEASUREMENT AND DARK-ADAPTATION IN A SUBJECT DEFICIENT IN CONE VISION 193

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